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ERRATA

In Volume 4, page 395, first paragraph, the values of "3.5 and 2.9 γ " and "3.4 and 2.9 γ "

should be replaced by "3500 and 2900 γ ", and "3400 and 2900 γ ", respectively.

In Volume 5, page 158, in Table I, the first paragraph of the text should read:

"choline chloride, 500 mg.;"

instead of

"choline chloride, 500 g.;"

ERRATA

In Volume 4, page 244, the footnote should read:

“potassium bromate”

instead of

“potassium bromide”

Volume 4, page 246, the first sentence of the last paragraph should read:

A bread containing 2.3% milk solids and 3% full fat soya flour was superior to a bread containing 6% skim milk solids.

Carotenoid Pigments in the Ocean Floor¹

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From the Scripps Institution of Oceanography of the University of California, La Jolla, California

Received April 18, 1944

INTRODUCTION

Two earlier contributions from this laboratory (Fox, 1937; Fox and Anderson, 1941) dealt briefly with the finding in ocean sediments of what might be called marine *paleobiochromes*, or natural marine pigments of great antiquity from a biochemical viewpoint, although perhaps from relatively recent deposits in the geological sense.

The present discussion is concerned with further investigations of these compounds, their probable origins, history, and chemical significance. These pigments are chiefly fat-soluble yellow, orange, and red members of the carotenoid class and greenish cleavage products of chlorophyll. They occur commonly in the presence of only small amounts of other lipid-soluble materials, and are regarded as "biochemical fossils" since the time of their burial may, in some strata, antedate the Christian era, the building of Stonehenge, or even the construction of the Egyptian Pyramids.

It is believed that further researches into the occurrence and nature of pigmentary compounds in natural deposits may yield valuable information regarding the biogenic origins of petroleum, coal, shale oil, and the like. In this connection, readers are referred to publications by Treibs (1934) and a review by Lemberg (1938).

Marine carotenoids and chlorophyllous compounds occur together in phytoplankton and other photosynthetic marine plants, and hence in suspended de-

¹ Contributions from the Scripps Institution of Oceanography, New Series No. 236.

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tritus, feces, marine sapropel, and mud. Carotenoids are also encountered abundantly in many chromogenic bacteria and fungi, and in the tissues (notably integument, liver, and gonads) of many animal species at all depths. Fauna living beneath the lighted zones of the sea are necessarily of carnivorous, detritophagous, or omnivorous habit. Their tissue carotenoids are therefore related metabolically to the polyenes supplied in (1) the bodies of their prey, (2) the incessant rain of algae and organic detritus from the waters above, and possibly (3) the activities of mud-dwelling chromogenic microorganisms as well.

Ingested carotenoids are, in turn (1) partly rejected unchanged from the alimentary tract whence they enter the constitution of bottom mud; (2) partly oxidized metabolically in various degrees; and (3) those stored in tissues may so remain until the animal dies or is eaten.

The continuous supply of organic material from plants and animals of the euphotic zone provides the ultimate food supply for organisms in the depths of the sea. Carotenoids are among the compounds which are less readily assimilated in the alimentary tracts of consumers, and they are less drastically modified by those animals which do assimilate and store them. Thus the manufacture and supply of carotenoids by diatoms, dinoflagellates and other photosynthetic plants, bacteria, fungi, and perhaps also by certain animal species, considerably exceeds the destruction of these pigments. Consequently, some areas of the ocean's floor constitute vast reservoirs for carotenoids and related compounds, which, because of prevailing reducing conditions in the mud proper, the absence of light and the low temperatures, are preserved in a buried condition for centuries of time. In deposits of hundreds or thousands of years' standing, it is doubtless possible that isomerization and other chemical changes may gradually have occurred, with or without microbiological intervention. A number of well known bacterial carotenoids, and a few others of previously unreported absorption spectra have been encountered. Some of the carotenoids met with would appear to have been stored unchanged for ages past. Examples are β - and α -carotene, fucoxanthin, and xanthophylls closely resembling lutein and zeaxanthin. In the light of very recent work by Strain, Manning, and Hardin (1944) it is believed that a considerable part of our sedimentary lutein-like xanthophylls may prove to be diadinoxanthin, extracted from marine diatoms and dinoflagellates, or possibly neodiatoxanthin, an isomer of diatoxanthin from diatoms. Likewise, it is suspected that the zeaxanthin-like xanthophylls encountered in mud are largely diatoxanthin and its isomers found by Strain, *et al.* in diatoms. Sulcatoxanthin, the anemone xanthophyll, is believed by Strain and his associates to be identical with peridinin from peridinians; and violaxanthin and taraxanthin, found respectively in pansy and dandelion flowers, find their marine counterpart in dinoxanthin and its experimental isomer neodinoxanthin, from marine dinoflagellates.

The presence of carotenoids and allied pigments in natural deposits has received attention by a few other workers (1, 17, 20, 2, 31).

This paper deals with the kinds, and in part with relative concentrations of carotenoids in increasingly deep layers of mud, representing suc-

cessively greater ages of deposits. Emphasis is given as well to the possible selective role of marine animals and microorganisms.

Mud samples were studied from regions off the southern California coast, from the Gulf of California, from Mission Bay, and from a freshwater body, Lake Hodges, in San Diego County.

GENERAL METHODS

Mud-surface sediments were collected with a mechanical snapper; cylindrical columns or cores of deeper lying material were secured with the special coring device of Emery and Dietz (1941).

The marine core-sections examined were invariably highly reducing media, numerous specimens evolving hydrogen or light hydrocarbon gases, accompanied by H_2S , when first collected. All samples were stored in tightly stoppered containers and kept in a cool dark place (often in a refrigerator) pending examination. For analysis, the samples were dried at room temperatures in vacuo, and the fat-soluble colored material was then extracted by repeated treatment with acetone or ethanol. The pigments were subsequently partitioned into (1) epiphasic or carotene-like fractions, selectively migrating to petroleum ether, and (2) hypophasic fractions preferentially soluble in 90% methanol. The hypophasic or xanthophyll carotenoids were finally driven into fresh petroleum ether by dilution with water. Both fractions were washed free of methanol, dried if necessary, and adjusted to known volumes. Aliquot portions of each were transferred to CS_2 for spectrophotometric estimation. A Bausch and Lomb visual spectrophotometer was employed for reading the absorption of all such pigment solutions. The wave length of $485 m\mu$ was selected, since the aggregated carotenes, as well as the mixed xanthophyll fractions, each showed maximal light absorption at or very close to that point. Epiphasic carotenoids were therefore estimated in " β -carotene equivalents," and hypophasic pigments usually in "zeaxanthin equivalents," in accordance with the apparent preponderating compound in each respective solution. The preponderating xanthophyll was in all probability the diatom pigment diatoxanthin, whose absorption maxima are identical with those of zeaxanthin, but whose specific absorption coefficients have not yet been determined (Strain, *et al.*, 1944).

Tswett chromatographic columns of $Ca(OH)_2$ or MgO were employed for separating individual carotenes from the original epiphasic fraction in petroleum ether; the xanthophylls were resolved into their components by similar adsorption on $CaCO_3$. Chromatograms were developed with benzene if necessary, and each pigment zone was finally eluted with petroleum ether containing small amounts of methanol (Zechmeister and Chohnoky, 1943). The individual pigments were transferred to CS_2 and examined with a Hartridge reversion spectroscopy or with the Bausch and Lomb instrument. For diagnostic purposes, it should be emphasized that the Hartridge spectroscopy was often found to give slightly higher maxima than were determined with the more accurate spectrophotometer.

Partition of epiphasic pigments between petroleum ether and 95% methanol

demonstrated the absence of all save occasional possible traces of cryptoxanthin. Treatment of epiphasic fractions with hot alcoholic KOH occasionally yielded traces of xanthophyll-like compounds, suggesting the original presence of small amounts of corresponding esters. The amounts were too small, however, to be quantitatively significant in this survey, and may indeed have been products of partial degradation. Likewise, heating hypophasic materials with alcoholic potash only rarely gave rise to faint traces of acidic pigments reminiscent of the astacene group.

RESULTS

Muds from the Gulf of California were far richer both in green chlorophyll derivatives and in carotenoids than were samples of strictly oceanic muds or sediments from nearer shore. Extracts of Gulf muds were usually deep brownish green or brownish yellow due to the presence of chlorophyllous derivatives (intact chlorophyll was never encountered among the mud pigments) and carotenoids, while pigment solutions from oceanic sediments were commonly golden or dirty yellow, yielding much smaller amounts of carotenoids and very little greenish material.

The present studies are confined to the carotenoids. Table I presents a summary of qualitative findings. The relative paucity of pigment allowed only a few classifying properties to be determined. We conducted the treatment as rapidly as possible, and rarely repeated chromatographic purification. Thus it was hoped to minimize alterations or losses by isomerization or oxidation.

Table II lists a number of carotenoids whose absorption maxima resemble those of some of our marine pigments. Thus, while it is not always possible to state that a given pigment encountered in marine mud is, say β -carotene, nevertheless its phasic, chromatographic, and spectroscopic properties may suffice to place it in Group III, which includes β -carotene, or, in the xanthophyll series, zeaxanthin, (or diatoxanthin), etc.

It will be noted in the final column of Table I that, although unusual carotenes resembling such compounds as torulene, flavorhodin, leprotene, or rhodopurpurin, are encountered occasionally, the chief carotenes in sediments belong to (epiphasic) Group III, the β -carotene class, with secondary representation of Group II or α -carotene. Similarly, in the xanthophyll series, while unusual compounds resembling glycimerine or astacene appear in certain samples, we encounter chiefly the hydroxy derivatives of the commoner carotenes, in that members of the zeaxanthin or diathoxanthin class (III) are the chief hypophasic constituents,

with the lutein (diadinoxanthin, neodiatoxanthin) or fucoxanthin group (II) in secondary prominence. In a few instances, xanthophyllic pigments seem to resemble carotenoids of two neighboring groups. In such cases, the assigned group-number represents the pigment's similarity in the position of its second (but chief) maximum; the position of this second maximum (*i.e.*, in the blue-violet) is the more important and more stable criterion.

It is of interest to note the frequent occurrence, in marine sediments, of xanthophyllic carotenoids whose chief absorption maxima in the violet region lie close to $480\text{ m}\mu$, intermediate between the positions of corresponding peaks of lutein or diadinoxanthin ($475\text{ m}\mu$) or fucoxanthin ($477\text{ m}\mu$) on the one hand, and zeaxanthin or diatoxanthin ($482\text{ m}\mu$) or cryptoxanthin and cynthiaxanthin ($483\text{ m}\mu$) on the other. This is true to a less extent of some of the carotenes, which thus resemble leprotene, while the corresponding xanthophylls are like antheraxanthin, petaloxanthin or sulcatoxanthin (peridinin). Structural isomerization (*e.g.* diatoxanthin \rightarrow neodiatoxanthin, according to Strain, *et al.*) is a conceivable factor in the production of some of these marine xanthophylls of Group III during vast periods of time. It is also to be recalled that closely similar xanthophyllic pigments have been encountered in marine invertebrates, *i.e.*, the zooxanthellae-bearing anemones, *Anemonia sulcata* (Heilbron, *et al.*, 1935) and *Cribrina xanthogrammica* (Strain, *et al.*, 1944), another anemone, *Metridium senile*, which is free from algal symbionts (Fox and Pantin, 1941), herbivorous and carnivorous echinoderms (Fox and Scheer, 1941), and occasionally in the liver of another carnivore, the octopus (Fox and Crane, 1942).

Members of this same class of pigments are to be found also in the feces of the California mussel, a consumer of suspended, finely divided marine detritus.

In addition, the results of some preliminary experiments on the bacterial decomposition of one of the commercially important giant kelps, *Macrocystis pyrifera*, are interesting. The fresh blades yielded, as the only major carotenoids, two pigments with absorption spectra closely resembling β - and α -carotenes respectively (although the pigments separated in reverse of the usual chromatographic order) and considerable amounts of fucoxanthin. Finely comminuted tissues of the plant were exposed to mixed bacteria in sea water at initial pH values of 7-8, and were thus incubated in the dark at prevailing room temperatures for periods of from 100 to 186 days. When analyzed, the particulate organic

TABLE I

Spectroscopic Properties of Carotenoid Fractions from Various Sediments

All pigments were examined in CS_2 with a Hartridge reversion spectroscope (H) or a Bausch and Lomb visual spectrophotometer (S). Epiphasic (e), hypophasic (h), and carotenoids passing through the columns (f) are indicated by serial numbers according to relative position on the chromatographic column of $\text{Ca}(\text{OH})_2$ or CaCO_3 respectively, from top downward (green and excessively dilute zones usually omitted); where colors of zones are recorded, gm. = green; grush. = greenish; or. = orange; pk. = pink; r. = red; ylo. = yellow. Where serial numbers are omitted, or followed by a dotted line, the fraction was too scanty for reliable spectroscopic observation.

Location and Sample	Depth fathoms	Core section inches	Average estimated age years	Chromatograph zone of epiphasic (e) or hypophasic (h) fraction	Absorption maxima in CS_2 , $m\mu$	Remarks	Provisional identification or group (see Table II)
Gulf of Calif. 27°44'N 110°50'W (XVI-8)	106	14-18	530	e-3	514.3; 484.1(H)		β -carotene
				e-4	513.4; 479.5(H)		leptotene(?)
				e-2	515.6; 482.8(H)		III
	54-58		1870	e-3	516.6; 481.2(H)		III
				h-2	506.2; 481.7(H)		II, III
				e-2	519.3; 483.8(H)		β -carotene
	102-106		3470	e-3	508.6; 479.8(H)		α -carotene
				h-2	506.9; 480.4(H)		II
				e-3	522.1; 485.4(H)		β -carotene
	134-136		4500	e-4	519.5; 483.6(H)		"
				e-5	517.0; 482.1(H)		"
				e-3	519.0; 483.1(H)		"
Gulf of Calif. 27°17'N 111°08'W (XVI-10)	930	8-12	330	h(whole)	512.4; 481.2(H)	sharp bands	antheraxanthin?
				h-3	507.2; 481.6(H)	diffuse bands	II, III
				e-2	517.2; 482.3(H)	"	III
	24-28		470	e-3	515.7; 480.1(H)		III
				h-1	512.1; 478.7(H)		II

Gulf of Calif. 27°38'N 110°49'W (XVI—27)	209	28-32	1000	h-2	509.9; 478.3(H)	blurred bands very sharp bands	fucoxanthin sulcatoxanthin or peridinin.
				h-3	516.7; 481.9(H)		
	2200	64-68		e-3	513.4; 478.9(H)	diffuse bands	leptotene “
				e-4	514.2; 478.3(H)		
				h-3	510.6; 480.3(H)	band I thin; band II broad	II, III rhodopurpurin III
				e-2(b)	517.6; 515.8; 481.4(H)		
				e-2(c)	520.3; 485.7; 455.5(H)		
				e-3	517.0; 480.7(H)		
				h-1	510.0; 479.0(H)	sharp bands; strong ab- sorption coeff.	leptotene fucoxanthin “
				h-2	509.1; 479.2(H)		
				h-3	515.7; 481.6(H)		
				h-6	510.4; 482.1(H)		
				e-2	512.6; 481.0(H)		
	90-100		3300	e-3	508.7; 521.6; 485.5(H)	torulene	III III
				e-4	514.8; 480.0(H)		
				e-5	516.0; 482.2; 453.7(H)	III III	III III
				h-1	513.8; 481.8(H)		
				h-2	511.8; 481.9(H)	sharp bands; strong abs. coeff.	petaloxanthin III
				h-3	512.8; 482.5(H)		
				h-4	514.3; 481.1(H)		
				h-1	512.9; 486.8(H)		
	108-112		3700	h-1	512.9; 486.8(H)	very sharp bands	zeaxanthin or diatoxanthin
				h-4	514.8; 480.7(H)		
						blurred bands	petaloxanthin?

TABLE I—Continued

Location and Sample	Depth fathoms	Core section inches	Average estimated age years	Chromatograph zone of epiphasic (e) or hypophasic (h) fraction	Absorption maxima in CS ₂ $m\mu$	Remarks	Provisional identification or group (see Table II)
Gulf of Calif. 27°54'N 111°42'W (XVI-49)	366	8-12	330	e-2	519.0; 483.4(H)		β -carotene
				e-3	518.7; 483.5(H)		β -carotene
				e-4	511.3; 477.2(H)	diffuse bands	α -carotene
		84-88	2870	e-2	506.6; 478.6(H)		α -carotene
				e-3	512.7; 478.0(H)	sharp bands	leptotene
				h(whole)	512.4; 480.0(H)	sharp bands	anthraxanthin?
		154-158	5300	e-2(a)	567.7; 522.3; 486.4(H)		torulene
				e-2(b)	ca 500 in pyridine (H)	acidic on hydrolysis	astacene(?)
				e-3	518.5; 481.2(H)	diffuse bands	III
				e-4	513.7; 480.0(H)	very strong abs. coeff.	III
				h-1	510.9; 480.6(H)	sharp bands	II, III
				h-2	506.5; 476.6(H)		II
				h-3	516.1; 481.2(H)		III
				Extr. I:			
	ca.	—	—	e-3	522; 487(H); 515; 485; 452(S)		β -carotene
Gulf of Calif. 28°39'N 112°59'W (XVI-222)	800			e-6	512; 483(H); 506; 481.5(S)	re-chromatographed	α -carotene
				e-7	512; 480(H); 508; 480(S)	Spectrum = 506; 478(S)	
				h-1	510; 481(H); 510; 480(S)		II, III

h-2	500 to 505(H): 507; 490 (S)		
h-4	507(H): 485 to 500 487 in pyridine (S) (S)	glycymerin?		
Extr. II:				
e-7	514; 480(H)	III		
h-3	500(H): 501(S)	Strong reactions with glycymerin? SbCl ₃ or H ₂ SO ₄ . Un- changed by hydrolysis treatment. Principal carotenoid in hypo- phase		
Channel Isl. re- 106				
gion off So.				
Calif. coast				
32°37'N				
117°21'W				
(S.L.600)				
e-1 grn.-ylo.	—			
e-2 lt. ylo.	—			
e-3 pk.	510; 485(S)	III		
e-4 pk.-or.	520; 485(S)	β-carotene		
e-5 grnsh.	—			
e-6 r.	—			
e-7 or.	565; 522; 493(S)	torulene		
e-8 ylo.	513; 482(S)	III		
e-9 or.	—			
e-10 ylo.	—			
f-1 ylo.	472(S)	?		
f-2 ylo.	485(S)	?		
f-3 or.	507; 477(S)	α-carotene		
f-4 or.	510; 477(S)	α-carotene		
h-1 ylo.	510; 484(S)			
h-2 r.	507; 485(S)			
h-3 ylo.	503; 488(S)	III		
h-4 r.	507; 485(S)			

TABLE I—Continued

Location and Sample	Depth fathoms	Core section inches	Average estimated age years	Chromatograph zone of epiphasic (e) or hypophasic (h) fraction	Absorption maxima in C ₂₅ mp	Remarks	Provisional identification or group (see Table II)
Channel Isl. region off So. Calif. coast	650	92-102	2400	e-1 pk. e-2 r.	515.4; 484.2(H) 564.9; 523.7; 492(H)		III torulene
32°33'N				e-3 or.	517.2; 480.4(H)		III
117°27'W				e-4 r.	517.0; 483.5(H)		β-carotene
(S.L.496)				e-5 or. (lost)	—		flavorhodin
				e-6 ylo.	503.1; 474.8(H)		III
				e-7 r.	515.1; 487.8(H)		flavorhodin
				e-8 or.	503.2; 473.8(H)		
				e-9 or.	—		
				e-10 ylo.	—		
				e-11 or.	511.7; 484.2(H)		III
				f-1 ylo.	510.6; 469.4(H)		?
				f-2 or.	505.7; 475.4(H)		α-carotene
				f-3 or.	512.6; 478.2(H)		α-carotene
				h-1 ylo.	509.4; 479.4(H)		fucoxanthin
				h-2 ylo.	514.1; 470.9(H)		?
				h-3 or.	516.6; 482.3(H)		III
				h-4 r.-or.	508.4; 469.0(H)		I, II
				e-8	513; 481(H)		I, II
				e-9	513; 479(H)		III
				h-1	502; 476(S)		III
				h-2	499; 472(S)		I, II
				h-3	500; 477(S)		I
						Blue color with 25% HCl; not hypophasic to 70% CH ₃ OH	I, II
Mission Bay (fine black intertidal sand)	Low tide level	6-24	—			No color with 25% HCl	?
Lake Hodges (fine black mud)	8	Snap sample from mud	—	h-4 e-3 e-6	485; 470(S) 471; 428(H) 516; 484.5(H)		β-carotene

material in the cultures had decreased greatly in weight and had been degraded from small shreds to a fine powdery condition. Cultures which had been rendered anaerobic for the final two-month period

TABLE II
Absorption Maxima (in $m\mu$) of some Carotenoids in CS₂
(From Strain, 1938; Lederer, 1935)

	Carotenes	Xanthophylls
Group I.....	Flavorhodin 503; 472; 441	Violaxanthin 500.5; 469; 440 Taraxanthin 501; 469; 441 Eloxanthin 502; 472 (Dinoxanthin; neodinoxanthin; neodiatinoxanthin) ³
Group II.....	α -Carotene 507; 476	Pentaxanthin 506; 474; 444 Lutein 508; 475; 445 Fucoxanthin 510; 477 (Diadinoxanthin; neodiatoxanthin) ³
Group III....	Leprotene 517; 479; 447 β -Carotene 511 ¹ ; 485 Echinenone ² (520); 488; (450)	Antheraxanthin 512.5; 481; 448 Petaloxanthin 514.5; 481 Sulcatoxanthin (Peridinin) 516; 482; 450 Zeaxanthin 517; 482; 450 Cynthiaxanthin 517; 483; 452 Cryptoxanthin 519; 483; 452 Pectenoxanthin 518; 486; 452 (Diatoxanthin) ³
Group IV....	Rhodopurpurin 550; 511; 479 Lycopene 548; 507.5; 477	
Group V.....	Torulene 566; 522; 491; 461	

¹ Listed by some writers as occurring as high as 521 $m\mu$ with certain instruments (see Smith, 1936).

² An epiphasic oxygenated carotenoid from echinoderms (Lederer, 1938).

³ Parenthesized names are those of newly described xanthophylls (Strain, *et al.*, 1944) whose absorption spectra in ethanol or petroleum ether place them in the indicated groups, but whose CS₂ absorption spectra have not been published.

yielded especially interesting results. No significant pH changes in these were manifest, although considerable amounts of H₂S were apparent, and motile bacteria were observed. β -Carotene remained in

apparently unchanged quantities, but fucoxanthin seemed to have been chemically changed in part to xanthophylls of Group III, spectroscopically similar to antheraxanthin and petaloxanthin, with absorption maxima (in CS₂) of 510, 480 m μ and 513, 481 m μ . In one 100-day aerobic culture, a xanthophyll resembling taraxanthin (or dinoxanthin, neodinoxanthin or neodiadinoxanthin discussed by Strain, *et al.*) was detected, with absorption maxima at 499, 470, 443 m μ . This was accompanied by a unique, unstable xanthophyll (477, 448 m μ).

In the anaerobic cultures, pigments resembling the antheraxanthin class appeared perhaps in partial replacement of fucoxanthin. Thus the preliminary anaerobic experiments, designed to simulate somewhat conditions in the ocean floor, resulted, through a microbiological agency, in a carotenoid picture similar to some which have been encountered in marine sediments.

In the foregoing connection, some observations of Clarke and Mazur (1941) on the microbial reduction of diatom lipids are of interest. Analyses of various diatom catches and cultures revealed a high preponderance of uncombined fatty acids over all other constituents of the lipid fractions. Furthermore, decomposition of diatom sludges by marine microorganisms over periods of 6 months yielded a great fall in the proportion of free acids, often with a marked increase in the hydrocarbon fraction. The data reveal higher proportions of hydrocarbons, in marine mud and in diatom sludges decomposed by bacteria under air or under nitrogen, than in fresh diatoms. Thayer (1935) observed that carotenoids and chlorophyll derivatives were among the most resistant protoplasmic components in the anaerobic decomposition of diatoms. He suggested that pigments may contribute, at least in a minor way, to the genesis of petroleum. ZoBell (1944) has considered the potential role of lipolytic bacteria in the genesis of hydrocarbons allied to those encountered in petroleum.

Table III lists values for epiphasic and hypophasic pigments in various sediments of different ages. The primary arresting fact is the high proportion of carotenes over xanthophylls in all sediments, as compared with representative marine plant and detrital materials (Table IV). At Stations S.L. 600 and S.L. 601, the total carotenoid content of marine sediment underwent a decrease with depth down to 4 feet, representing an aggregated accumulation of more than 7 centuries.⁴

⁴ Geological information from Professor Francis P. Shepard, personal communication.

TABLE III

Quantities of Carotenes and Xanthophylls in Marine Sediments of Different Ages
 Carotenes are expressed in terms of β -carotene equivalents, xanthophylls in zeaxanthin units unless otherwise specified

Location and Sample	Depth	Core section	Av. estimated age	Carotenes mg./ 100 g. (dry wt.)	Xanthophylls mg./ 100 g. (dry wt.)	Total carotenoids mg./ 100 g. (dry wt.)	Carotenes per cent
Channel Isl. region off So. Calif. coast 32°37'N 117°21'W (S.L. 600)	106 fathoms	Surface grab sample	— years	0.13	0.04	0.17	76.5
Same as S.L. 600 (S.L. 601)	106	1-12	15-180	0.20	0.04	0.24	83.2
		12-24	180-360	0.05	0.01	0.06	83.2
		24-36	360-540	0.04	0.01	0.05	80.0
		36-48	540-720	0.02	0.01	0.03	66.6
Off Pt. Loma, Calif., 32°37.6'N (Fox 118°09'W 1937)	1096	1-4	—	0.07	0.13	0.20	35.0
Channel Isl. region off So. Calif. coast 32°33'N 117°27'W (S.L. 496)	650	96-102	2500	0.24	0.05	0.29	83.0
Gulf of California 27° 41'N 110° 55'W (Fox and Anderson, 1941)*	364	180-195	6000	0.42	0.19 (lutein units)	0.61	69.0
Mission Bay (fine black intertidal sand)	Low-tide level	—	—	—	—	0.01	(mostly carotenes)
Lake Hodges (fine black mud)	8	Surface grab sample	—	0.11	0.18	0.29	37.8

* The figures in this table are corrected; those published in the earlier paper cited (p. 335) are 10-fold too high due to a misplaced decimal.

The proportion of carotenes in total carotenoids rose from 76.5% at the mud surface to 83% in the first 2 feet of the core; the succeeding 3 and 4-foot depths of sediment showed a steady decrease in the preponderance of hydrocarbon types over oxidized carotenoids, although even at the 4-foot depth, wherein the total carotenoid content was about half that at the surface, carotenes still constituted 66%. Local differences are

Quantities and Relative Proportions of Carotenes and Xanthophylls in Marine Plants and Other Marine Materials

Values are expressed in mg. per 100 g. dry material unless otherwise specified.

Material	Carotenes	Xanthophylls	Total	Carotene per cent	Reference
<i>Prorocentrum micans</i> . . .	0.26	2.26	2.52	10.3	Scheer, 1940
<i>Nitzschia closterium</i> . . .	65.9	578.5	644.4	10.3	Pace, 1941
" "	—	—	270, 296	Fox (unpubl.)
Sea-water detritus (from sea-water settling reservoir via lead pipe to laboratory)	—	0.123 mg. (from 4000 liters)	18.3	Fox, 1937
Mussel feces	3.3	51.0	54.3	6.1	Fox, <i>et al.</i> , 1936
" "	2.0	12.5	16.0	Fox (unpubl.)
Feces of mussels on <i>Prorocentrum</i> diet . . .	0.75	4.38	5.13	14.6	Scheer, 1940
<i>Fucus</i> sp.	8.9	25.6	34.5	25.7	Willstätter and Page (from Zechemister, 1934)
<i>Dictyola</i> sp.	5.7	31.3	37.0	15.4	"
<i>Laminaria</i> sp.	0.6	11.9	12.5	4.8	"
<i>Rhizosolenia styliformis</i> + <i>Biddulphia sinensis</i>	35.0	65.0	100.0	35.0	Gillam, El Ridi, Wimpenny, 1939

exemplified by data collected from a core taken at Station S.L. 496 at a depth of 650 fathoms. Here at a depth of some 8 feet (estimated age of deposit about 2500 years), the total carotenoid content of vacuum-dried sediment even exceeded that of the first foot of the other core, *i.e.*, 0.29 mg.%, the proportion of carotenes being 83%. Black, reducing sediment (containing H_2S as in the other samples) collected from the low-

TABLE V

Approximate Quantities of Xanthophylls and Carotenes in Various Marine Species
Expressed in mg. per 100 g. wet tissue

Species	Xantho- phylls	Caro- tenes	Approx. carotenes %	Reference
<i>Fishes</i>				
<i>Fundulus parvipinnis</i>	0.77 to 1.2	0	0	Sumner and Fox (1933, 1935a)
<i>Girella nigricans</i>	ca 1.4	0	0	Sumner and Fox (1935b)
<i>Gillichthys mirabilis</i>	0.41 to 0.62	0	0	Sumner and Fox (1933)
<i>Cymatogaster aggregatus</i> ..	ca 0.27	0	0	Young and Fox (1936)
<i>Hypsypops rubicunda</i>	very concen- trated in skin	0	0	Fox (1936)
<i>Mollusks</i>				
<i>Mytilus californianus</i>				
male body.....	2.18	0	0	Scheer (1940)
male gonad.....	6.05	0	0	"
female body.....	4.83	0	0	"
female gonad.....	11.34	0	0	"
<i>Paroctopus bimaculatus</i>				
ink.....	0.55 to 0.7	0	0	Fox and Crane (1942)
<i>Echinoderms*</i>				
<i>Dendraster excentricus</i> ..	0.049	0.157	76	Fox and Scheer (1941)
<i>Strongylocentrotus fran- ciscanus</i>	0.025	0.170	87	" " " "
<i>Strongylocentrotus pur- puratus</i>				
female intestine.....	6.04	2.99	33	" " " "
female gonad.....	0	2.00	100	
male intestine.....	5.65	2.41	30	
male gonad.....	0	0.69	100	
<i>Lytechinus pictus</i>				
whole females.....	0.35	0.49	58	" " " "
whole males.....	0.43	0.87	67	
female intestine.....	4.18	4.48	52	
female gonad.....	0.28	0.86	75	
female test.....	0.01	0.01	50	
male intestine.....	1.52	3.38	69	
male gonad.....	0.53	4.48	90	
male test.....	0.01	0.01	50	
<i>Stichopus californicus</i> ..	0.015	0.014	48	" " " "
<i>Astropecten californicus</i> ..	0.72	0.044	5.8	" " " "

TABLE V—Continued

Species	Xanthophylls	Carotenes	Approx. carotenes	Reference			
<i>Patiria miniata</i>	0.86	0.05	5.5	Fox and Scheer (1941)			
<i>Pisaster ochraceus</i>							
skeleton + skin	0.24	0.23	49	"	"	"	"
pyloric caeca	11.45	0.56	4.7				
<i>Pisaster giganteus</i>							
skeleton + skin	0.90	0.07	7.2	"	"	"	"
pyloric caeca	1.37	0.40	22				
<i>Ophiopteris papillosa</i> . . .	>3.39	0	0	"	"	"	"
<i>Ophiothrix spiculata</i> . . .	>1.60	0	0	"	"	"	"
<i>Ophiothrix rudis</i>	>1.40	0	0	"	"	"	"
<i>Annelid</i>							
<i>Thoracophelia mucronata</i>	0	0.38	100	Sumner and Fox (1933)			
<i>Anemone</i>							
<i>Metridium senile</i>							
red variant	14.96	0**	0**	Fox and Pantin (1940)			
white variant	1.76	0	0	"	"	"	"
orange variants }	(highly variable, but carotenes in minority when present)			"	"	"	"
brown variants }							

* It should be emphasized here that, among the echinoderms, notably such members of the echinoid class as the urchins *Strongylocentrotus* and *Lytechinus*, the ketonic carotenoid *echinenone* was demonstrated by Fox and Scheer (1941) to constitute a considerable portion of the epiphasic pigments, placed therefore in the hydrocarbon group with the carotenes. Echinenone seemed to be present also in the holothurian, *Stichopus*, and in one asteroid, *Astropecten*. The presence of echinenone in the carotene fraction would of course diminish the relative proportion of true carotenes listed in certain echinoid tissues in the above table.

** Occasional red individuals were found to contain a little carotene.

tide shoreline in Mission Bay yielded carotenoids in amounts of only 0.01 mg.%. The lake sample yielded but four epiphasic carotenoids against 14 such fractions in the marine material; β -carotene was present in both kinds of deposit. The hypophase from the lake mud yielded only a lutein-like xanthophyll, while in the marine core (S.L. 601) the four chromatographic zones from the hypophase were dissimilar from this pigment (Table I).

A survey of the carotenoids present in some representative marine plants and animals reveals a striking reversal in the ratio of carotenes

to xanthophylls encountered in marine sediments. For in the green plants of the sea, the oxygenated class of carotenoids preponderates greatly over the hydrocarbon class (Table IV), and the same seems to be true of the great majority of marine animals. Indeed, xanthophylls are the exclusive carotenoids of some fauna, while we have encountered to date only one marine animal species, *i.e.*, the worm *Thoracophelia*, which stores only carotenes (Table V).

Table V shows a list of several marine animal species, most of which were found to contain xanthophylls only or in preponderance. Attention is called also to the important investigations of Lederer (1938), who examined the carotenoids of an extended list of organisms, and found, in tissues of numerous species of fish, tunicates, mollusks, and crustaceans, that oxygenated carotenoids were greatly preponderant, and in some instances the only polyenes present. In the gonads of the sea-urchin *Strongylocentrotus lividus*, Lederer encountered appreciable amounts of α - and β -carotene, accompanied by echinenone and a number of xanthophylls, predominantly the newly described xanthophyll penta-xanthin. In the sponges *Suberites domuncula* and *Ficulina ficus* he suspected the chief carotenoids to be members of the hydrocarbon group, *i.e.*, torulene, α -, β - and γ -carotene.

Karrer and Solmssen (*cf.* Lederer, 1938) obtained astacene from the sponge *Axinella crista-galli*, while Drumm and O'Connor (1940) found, in another sponge, *Hymeniacidon sanguineum*, no astacene, but echinenone and γ -carotene.

DISCUSSION

A. Approximate quantitative relationships

We may now draw some provisional conclusions regarding the fate of carotenoids in the sea. Resulting inferences may apply to channels of diagenesis involving hydrocarbons and other relatively refractory organic materials of marine sediments.

Krogh (1934) provides the average figure of 5.4 g. of total organic matter per cubic meter of sea water in the ocean as a whole. Trask (1939) records the approximate value of 2.5% of organic matter in near-shore sediments. He considers the average rate of sedimentation to be about 1 cm. in 25 years, and the density of the deposited material to be about 2.0. He points out that this would represent some 20 g. of organic matter accumulating on each square meter of bottom per year, or 2% of the estimated 1000 g. of organic compounds assumed to have been

synthesized over the annual period in the average 2000 fathoms of water above each square meter of ocean floor.

The concentration of pigments in ocean waters has not been estimated in an accurate way, but an approximation may serve to indicate the order of magnitude. Gillam, El Ridi, and Wimpenny (1939) provide data which indicate a general average of 0.0076 mg. of carotenoids per cubic meter of North Sea water. However, their use of a Hensen silk net of 60 meshes per inch would allow much of the very fine material to escape analysis. The additional stated fact that astacene was not measured would add to the departure from true values.

The occurrence of carotenoids and chlorophylls in certain ratios among marine algae (Pace, 1941; Zechmeister, 1934), and the estimations of Graham (1943) and others of green pigments in ocean samples might suggest the use of such indices as rough approximations of parallel carotenoid content. However, chlorophyll is readily decomposed by oxidation, hydrolysis, or other processes, whether in ordinary chemical ways or at the agency of bacteria or herbivorous animals. Therefore, serious inaccuracies might be involved in any attempts to estimate carotenoids in terms of unidentified and highly variable green pigments grouped together as "chlorophyll."

No extensive carotenoid determinations have been carried out on paper-filtered marine detritus, but some analyses have been made of fecal material from the California mussel, which derives its nutrition by filtering out and swallowing finely particulate and colloidally suspended marine detritus (*cf.* Scheer, 1940; Fox and Coe, 1943). Its feces often yield small, sometimes negligible quantities of chlorophyll derivatives, but never fail to contain varying but appreciable amounts of carotenoids. These have been estimated in a number of fecal samples. From Table IV, for example, a rough average value might be taken as about 24 mg.%, equivalent to about 80 mg. per 100 g. of fecal *organic* matter (Fox and Coe, 1943), of which carotenes were present in about the proportion often encountered in phytoplankton, *i.e.*, 15% of total carotenoids. Analysis of mussel fecal material has shown that about 12% of the organic matter may be lipid; thus the concentration of carotenoids in *lipids* of marine detritus would approach 0.66%. Bearing in mind that the quantities of carotenoids in marine plankton and detritus must be highly variable with season, locality, and other environmental factors, and that some of the xanthophyllic pigments ingested are assimilated by the mussel (Scheer, 1940), we may nevertheless

utilize carotenoid values in mussel feces as representative of the order of magnitude in materials that constitute the source of organic matter in bottom sediments.

The approximate data should reveal the general order of magnitude applying to the standing crop, the new crop, and the fossil crop of organic matter and carotenoids in the sea, as follows.

Standing crop: If the average quantity of organic matter is 5.4 g. per cubic meter (Krogh), and the average quantity of carotenoids 80 mg. per 100 g. organic matter, then a column of ocean water one square meter in cross section and 2000 fathoms (*i.e.*, 3650 meters) high should contain some 19,710 g. of organic matter, including about 2365 g. of lipids, and 16 g. of carotenoids (14 g. of xanthophylls and 2 g. of carotenes).

Annual crop: If, according to Trask, 1000 g. of new organic matter are synthesized per year in a column of ocean water of the above dimensions, this value would correspond to approximately 120 g. of lipids, including 0.8 g. of carotenoids (*i.e.*, 0.68 g. of xanthophylls and 0.12 g. of carotenes).

Fossil crop: Since no analytical studies could be made upon the composition of the uppermost few millimeters of mud, no information is available regarding the *annual* fossil crop. Some comparisons may be drawn, however, between the standing crop of suspended and that of long buried materials. A value of 0.25 mg. carotenoids per 100 g. dry sediment represents, on the basis of Trask's estimated 2.5% of organic matter in such muds, about 10 mg. carotenoids per 100 g. organic matter. We found about 0.05% of lipid material in samples of marine sediments, or approximately 2% of the organic residue. This would mean 500 mg. carotenoids (*i.e.*, 400 mg. carotenes and 100 mg. xanthophylls) per 100 g. sedimentary lipids.

In resumé, these rough approximations lead to some interesting provisional conclusions. If the organic fraction of marine detritus, represented by mussel-feces, contains 12% lipids, 68 mg.% xanthophylls and 12 mg.% carotenes, and if the organic portion of bottom sediments commonly yield 2% lipids, 2 mg.% xanthophylls, and 8 mg.% carotenes, this process of early fossilization would represent a loss of 83% in lipids, about 97% of xanthophylls, and only 33.3% of carotenes. But, whereas the lipid fraction would appear to have undergone a decrease of 83% in its xanthophyll content, *i.e.* from 566 mg.% down to 100 mg.%, the carotenes seem to have remained more refractory than either the xantho-

phylls or the lipids themselves, for their concentration of as much as 400 mg. % in sedimentary lipids is greater by some 4-fold than their estimated concentration in the lipids of suspended detritus. It is perhaps too early to extend into a generalization this apparent sparing of hydrocarbon carotenoids at the expense of the other lipids.

B. Implications Relative to Petroleum Formation

Marine lipid materials are commonly regarded as potential fore-runners of petroleum compounds. With this in mind, we may make a brief comparison between lipid-soluble components in the marine sediments which we have examined and the oil content of earth in representative oil wells.

One cubic meter of marine mud weighing 2000 kg. or 4400 lbs. contains approximately 0.05%, or 1 lb. of lipid-soluble constituents per ton. A good oil well is expected to bear at least 20 gal. or about 125 lbs. of oil per ton of solids. Representative marine sediments would therefore appear to bear roughly 1% of lipids potentially contributory to the genesis of natural oil. In spite of such a low figure, it is conceivable that some sediments may give up any excess oily material which then finds its way through fissures into pockets or masses. As an example, one marine sediment core examined in this laboratory yielded both chlorophyll degradation products and carotenoids at the 6 inch, 74 inch, and 80 inch levels (representing strata of 600, 7400, and 8000 years old respectively). The lipid contents of these three core-sections were 0.06%, 0.05%, and 0.04% by dry weight respectively. But an intermediate section of the same core, 44 inches beneath the mud surface (estimated age 4000 years), yielded the ten-fold quantity of lipid material. This sample contained greenish chlorophyllous material of the same spectral properties as that encountered in the other core sections, but, unlike them, bore no trace of carotenoids. Instead it yielded a pigment of blue-green fluorescence, identical in chromatographic behavior and spectral absorption with a pigment obtained from a sample of crude California petroleum (Fox and Anderson, 1941). The absorption spectrum of this pigment (in CHCl_3) agreed closely with that of a California asphalt pigment (in pyridine-ether) isolated by Treibs (1934).

Continued attempts to correlate findings of plant porphyrins, total lipid-soluble materials and carotenoids in marine sediments with similar studies on oil-bearing deposits are of manifest importance.

C. Qualitative Features

The properties of numerous carotenoids in marine sediments suggest their derivation directly from marine photosynthetic plants. Such pigments are the commoner carotenes, fucoxanthin, and certain newly described phytoplankton xanthophylls spectroscopically similar to zeaxanthin and lutein. Sulcatoxanthin, or peridinin, is common in marine mud. Animal carotenoids resembling glycimerin and astacene are occasionally encountered, while another group of pigments found characteristically in saprophytic plants suggest sedimentary contribu-

tion by such organisms. Torulene (from red yeasts) and other carotenes such as rhodopurpurin and flavorhodin (both from *Rhodovibrio* bacteria) are examples. It is noteworthy that these *hydrocarbon* pigments, ordinarily encountered in *Rhodovibrio* were not accompanied by xanthophyllic derivatives such as rhodoviolascin or rhodopin found in the same group of organisms.

Several chemical and biochemical agencies may combine in effecting the more rapid destruction of xanthophylls than of carotenes in the ocean's floor. In the first place, xanthophylls are more sensitive than are carotenes to atmospheric oxygen. In solutions or in solid form they more readily undergo autoxidation with bleaching (Willstätter and Mieg, *cf.* Zechmeister, 1934). The prominent marine xanthophyll, fucoxanthin and its isomers are especially susceptible to alteration by heat or alkalis (Strain, *et al.*, 1944) and may be bleached or otherwise altered by long standing. Secondly, it is known that the majority of marine animals investigated to date store chiefly xanthophylls rather than carotenes in their tissues. Exceptions may be encountered among bottom-dwelling worms, sponges, and echinoids. The xanthophyll-selecting animals doubtless oxidize some of the assimilated carotenoid instead of storing all of it. Filtering or bottom-feeding detritophagous animals which selectively assimilate xanthophylls, rejecting carotenes in their feces, could thus manifestly implement a gradual preponderance of the hydrocarbon type over the oxygenated type of carotenoid in marine sediments.

Finally, as has been suggested, microorganisms living in the anaerobic marine muds may be able to reduce certain xanthophylls to a lower oxygen content, or even to carotenes.

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SUMMARY

1. Sediment samples of the ocean floor contain substantial amounts of carotenoids and chlorophyllous degradation products laid down many centuries ago.
2. In contrast to green marine flora, likewise nearly all marine fauna

investigated, as well as suspended detritus of the sea, all of which yield xanthophyllic compounds in great excess over the carotene type, the relative proportions of these two carotenoid classes are strikingly reversed in marine muds.

3. Among the polyene hydrocarbons, β -carotene consistently seemed to preponderate, with α -carotene in secondary prominence, and various unusual carotenes, associated with bacteria, fungi, and certain sponges and ascidians, in lesser quantities and of more sporadic appearance.

4. Of the xanthophylls, zeaxanthin or more probably diatoxanthin, appeared to be the chief representative, followed by other alcoholic polyenes similar to antheraxanthin, petaloxanthin, sulcathoxanthin, lutein, fucoxanthin, and other xanthophylls described as characteristic of marine phytoplankton.

5. Of possible agencies operative in the selective storage of the hydrocarbon types over the oxygenated class of carotenoids in marine materials, several tentative suggestions are offered: (1) greater rates of autoxidation of xanthophylls than of carotenes in regions containing dissolved oxygen; (2) selective assimilation of xanthophylls and fecal rejection of carotenes by the majority of marine animals; (3) possible chemical reduction of xanthophylls to polyene hydrocarbons by certain marine bacteria in anaerobic environments.

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Biotin in Fermentation, Respiration, Growth and Nitrogen Assimilation by Yeast

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INTRODUCTION

Comparatively little is known of mechanisms by which trace organic dietary essentials influence the growth of various organisms, and of the possible direct metabolic roles these growth factors may play in fermentation, respiration, growth, or assimilation. Among the vitamins, thiamine, riboflavin, and nicotinic and pantothenic acids have been most definitely identified with readily measurable metabolic or chemical processes. The present work is an attempt to investigate biotin from this standpoint, and was undertaken as a result of the finding that biotin was identical with not only coenzyme R (1, 2, 3) but also with vitamin H, the anti-egg-white injury factor (3, 4, 5). This latter demonstration placed biotin among the animal vitamins, established vitamin H as a member of the vitamin B complex, and was of noteworthy significance in connecting plant and animal economy, as is attested by the many directions which research on biotin has taken since the demonstration of its connection with animal metabolism.

The discovery over a decade ago of coenzyme R (6, 7) as a factor essential for the growth of various legume root nodule bacteria, and as a heat-stable, dialyzable compound required in the respiration of these organisms, constituted one of the earliest demonstrations that the growth-stimulating property of an essential dietary substance was connected with a definite, readily measurable, metabolic process. In these early studies it was shown that various rhizobia grown with limiting amounts of coenzyme R had relatively low rates of respiration, and

that the addition of traces of coenzyme R concentrates resulted in a several-fold increase in respiration rate per unit weight of organism. This respiration increase required one to several hours for completion, and preceded any appreciable increase in cell number or dry weight.

The essentially universal distribution of biotin in living matter, its widespread occurrence as an essential growth factor, the fact that it is one of the most potent organic growth substances known—being detectable with rhizobia at less than 10^{-12} parts—and its aforementioned, demonstrated role in respiration of rhizobia, all make it very desirable to investigate the nature of its action not only in growth and in respiration but in particular to see whether it may also be demonstrated to play a role in the more intimate processes of fermentation or of assimilation of carbon, nitrogen, phosphorus, etc. For this purpose yeast is especially suitable since, in addition to the aerobic respiratory metabolism exhibited by rhizobia, yeast displays a highly fermentative and anaerobic mode of life that has already been studied by many investigators in great detail, and its assimilation processes are readily measurable. A preliminary account of such work with biotin-deficient yeast has been reported by us earlier (8); detailed studies are described here.

METHODS

In order to study the effect of biotin upon the metabolism of yeast, it was necessary to grow the organisms under conditions such that biotin was the limiting factor. This was accomplished by seeding a small quantity of yeast into the following medium used for the determination of biotin (9):

KH_2PO_4	2.0 g.	<i>l</i> -aspartic acid.....	0.1 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.25 g.	inositol.....	5.0 mg.
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.25 g.	β -alanine.....	0.5 mg.
H_3BO_3	1.0 mg.	thiamine.....	0.020 mg.
ZnSO_4	1.0 mg.	pyridoxin.....	0.020 mg.
MnCl_2	1.0 mg.	c.p. biotin-free sucrose.....	20 g.
TiCl_3	1.0 mg.	$(\text{NH}_4)_2\text{SO}_4$	3 g.
FeCl_3	0.5 mg.	H_2O	to 1 liter
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.1 mg.		
KI.....	0.1 mg.		

Steam-sterilized thiamine was added after the autoclaving of the rest of the medium. Biotin-free sucrose could be prepared by mixing 6–8 g. of charcoal (such as Darco) per 100 cc. of stock 40% sucrose solution, and, after 15 minutes standing at room temperature, filtering with suction through a thin layer of diatomaceous earth.

Maximum growth in the foregoing medium could be increased 50 to 100 per cent by addition of 0.5 cc. hydrolyzed vitamin-free casein (Smaco) per 100 cc. medium

(= 0.05 per cent casein hydrolyzate). Change in pH during heavy growth could be prevented by addition of 0.1 *M* succinate at pH 4.5 to 5.0 or 0.05 *M* citrate at pH 4.0. In the present studies neither casein nor succinate nor citrate buffer was used, except in certain standard bioassays.

The yeast employed, *Saccharomyces cerevisiae*, Fleischmann Strain 139, did not grow appreciably unless biotin was added to the growth medium. The addition of as little as 10^{-3} γ /cc. of crystalline biotin or its crystalline methyl ester permitted maximum growth with respect to biotin. Addition of about 5×10^{-5} γ /cc. yielded half-maximum growth. 100 cc. of the growth medium containing 3×10^{-6} γ biotin/cc. were put in sterile 250 cc. Erlenmeyer flasks and inoculated with 0.25 mg. dry weight of fresh yeast grown on wort or peptone agar slants. After 20 hrs. at 30° C. this yeast had grown to about 30 mg. dry weight, some 20–25% of the growth obtained with ample biotin. Such biotin-deficient yeast contained 0.01–0.05 γ biotin/g. dry matter, as compared with 2–4 γ /g. for our normal standard yeast. The yeast growths studied were washed twice by centrifugation, suspended in the salt solution of the growth medium at pH 4.2, and glucose added. The yeast dry weight was ordinarily determined from turbidity measurements of fresh yeast and read from a calibrated turbidity reading—dry weight curve. Biotin determinations on yeast were made after 2 hours' hydrolysis in 5% H_2SO_4 followed by neutralization of the solutions to pH 4.

The measurements of respiration (oxygen consumption) and of fermentation (carbon dioxide production) were made at 30° C. by means of the usual constant-volume, Warburg-Barcroft manometers, with and without alkali in the vessel insets, respectively. The oxygen consumption was ordinarily so small compared with the fermentation that it was sometimes neglected in calculating aerobic fermentation. In measuring oxygen consumption larger amounts of yeast were often used. The presence of alkali in the vessel insets involved measurements of oxygen consumption at somewhat lower pressures of CO_2 than those involved when measuring fermentation, and while this factor is important with animal tissues, the results reported here for yeast are not known to be affected by it. The pressure of CO_2 in the gas-phase of vessels containing alkali in the insets was of the order of 0.01 atmosphere, only slightly less than that in air.

Ammonium sulfate and biotin were ordinarily added from vessel side arms after temperature equilibration. In some experiments in which it was desired to know whether a dry weight or cell number increase had taken place, the yeast was removed from the Warburg vessels at the end of the experiment, and the dry weight determined from calibrated turbidity measurements on the Klett-Summerson photocolimeter, the cell count with a bacterial hemocytometer. Supernatants of centrifuged yeast cultures were measured for ammonia with Nessler's reagent, the readings being made on spot plates before secondary darkening due to reaction with glucose.

EXPERIMENTAL

The "biotin effect"

With a yeast grown at inadequate levels of biotin, the assumption may be made that the metabolic processes in which biotin most directly

participates. will be depressed and will show the most immediate response to the addition of biotin. In our first experiments we merely added biotin to unwashed yeast cultures which had ceased growing due to insufficiency of biotin, and then observed the effect of added biotin on the rates of fermentation, respiration, and growth. The rates of fermentation and respiration of such biotin-deficient yeast were much lower than normal, ranging from $\frac{1}{20}$ to $\frac{1}{10}$ of the rates in our normal standard yeast, and they remained constant for many hours if no biotin was added.

It was found that the aerobic and anaerobic rates of fermentation commenced to increase within a few minutes after the addition of biotin, whereas respiration did not rise appreciably until after the first hour. The turbidities of the yeast both with and without the added biotin remained unchanged during two to three hours' observation. Since increase in cell number, cell size, or cell opacity should result in an increased turbidity, we conclude that no appreciable growth, as judged by any of these criteria, had occurred within a sensitivity of a few per cent.

Observations with washed yeast suspensions gave results similar to those with the yeast cultures, providing phosphate, glucose, and ammonia were present, as indicated in Fig. 1.

The increase in rate of fermentation (or respiration, growth, etc.) elicited by addition of biotin to biotin-deficient yeast, and requiring some time, usually hours, for its full development, will for convenience hereafter be referred to as the *biotin effect*. The *biotin effect* does not here refer to any stimulated rate established completely and immediately, within a few minutes at most.

Evidently the order of response on addition of biotin was fermentation, respiration, and finally growth. This clear-cut kinetic separation of the effects of biotin in these three processes indicates that, whatever role biotin performs in metabolism, it is more intimately linked with fermentation than respiration or growth. This can mean that biotin is either an active intermediate in the known fermentation chain, or that it affects processes which may be regarded as preceding the increases in fermentation.

The influence of ammonia on the biotin effect

Allison and Hoover (7) found that coenzyme R had no effect on the respiration of coenzyme-R-deficient rhizobia unless assimilable nitrogen

was also present. The same was found to be true of the effect of biotin on the respiration and fermentation of biotin-deficient yeast. In Fig. 1 are shown the effects of separate and joint additions of biotin and ammonia on the rates of respiration and of aerobic and anaerobic fermentation. It is evident that unless both biotin and ammonia were present, there was no change in metabolism during the two hour experimental period. As in the experiments with unwashed yeast, the rates of aerobic and anaerobic fermentation began to rise within a few minutes after the addition of biotin, while corresponding increases in respiration commenced after about an hour. There was again no appreciable change in the dry weight of the yeast in the two hours. Considerable amounts of ammonia were taken up from the solution by the yeast in the presence of biotin, but none in its absence, as will become evident in a subsequent section.

The influence of biotin and ammonia concentration

The *biotin effect* as a function of initial biotin and ammonia concentrations is given in Table I. Biotin and ammonium sulfate were added from side arms in anaerobic Warburg experiments with, in one series, biotin, and in the other series, ammonia, in excess. It is seen that with increasing amounts of biotin or ammonia respectively, the other being in excess, the *biotin effect* rises to a maximum. No kinetic analyses of these curves were made because the concentrations of biotin and ammonia in the medium undoubtedly changed during the experiment. They do serve, however, to define the upper limits of the concentrations of ammonia and biotin which have the maximal effect on metabolism. It is of interest that the biotin concentration which gave half the maximum effect (0.5×10^{-4} γ /cc.) is essentially the same as that which gives half-maximum growth of yeast under the conditions used by us for biotin assay.

Biotin and ammonia "pretreatment"

It is quite evident that both ammonia and biotin are necessary for the *biotin effect*. We may now inquire as to what temporal relation may be involved between the interaction of biotin and ammonia, *i.e.*: Can either one exert any observable effect in the absence of the other, or must both be present together for any measurable influence on metabolism to result? One method to determine this is to add either the ammonia or the biotin first, and then after some time add the other. The results

of such an experiment on anaerobic fermentation with biotin-deficient yeast are given in Fig. 2. When the yeast had been incubated with ammonia for two hours, and biotin then added, the curve was practically a repetition of the curve when both were added at first. The ammonia, therefore, had no definitely demonstrable effect in the absence of biotin. When, however, the yeast was first incubated in the presence of biotin, the result was quite different. There was, as usual, no change in fer-

TABLE I

The Biotin Effect as a Function of the Concentration of Biotin and Ammonia

γ $\text{NH}_3\text{-N/cc.}$	I			Biotin Effect (%)	
	γ biotin/ cc. 10^4	Rate at 1st hr. $\text{Q}_{\text{CO}_2}^{\text{N}_2}$	II Rate at 2nd hr.	(I - 9.5) 100	(II - 9.5) 100
				9.5	9.5
500	37	23	40	142	322
25	37	21	30	126	216
5	37	19	28	100	195
2	37	18	25	89	163
0	37	17	22	79	132
0	37	10	10	5	5.3
500	185	24	41	153	331
500	18.5	23	40	142	322
500	5.6	21	39	121	310
500	1.85	20	37	110	295
500	0.74	16.5	30	73.8	216
500	0.37	12.5	20	31.3	110
500	0.185	10.5	14	5.3	47.5
500	0.09	9.5	11	0.0	16.7
500	0.0	9.0	9	-5.3	-5.3

Yeast grown at a biotin concentration of 3.7×10^{-6} $\gamma/\text{cc.}$, washed, and resuspended in glucose-salt solution. The indicated amounts of crystalline biotin and of ammonium sulfate were added at zero time and the rates of an aerobic fermentation followed by the Warburg method. The initial $\text{Q}_{\text{CO}_2}^{\text{N}_2}$ was 9.5 in all cases. The experiment was run in nitrogen gas at 30°C .

mentation rate under the influence of biotin alone, but, with the addition of ammonia, there was both an immediate initial increase in rate and a more rapidly established *biotin effect* than in the control curve. This indicates that biotin entered the yeast and exerted some influence in the absence of added ammonia, even though this influence did not become evident until the ammonia was added; of course, unobservable internal ammonia turnover may have been involved. In a later paragraph it will be shown that under our experimental conditions ammonia caused a

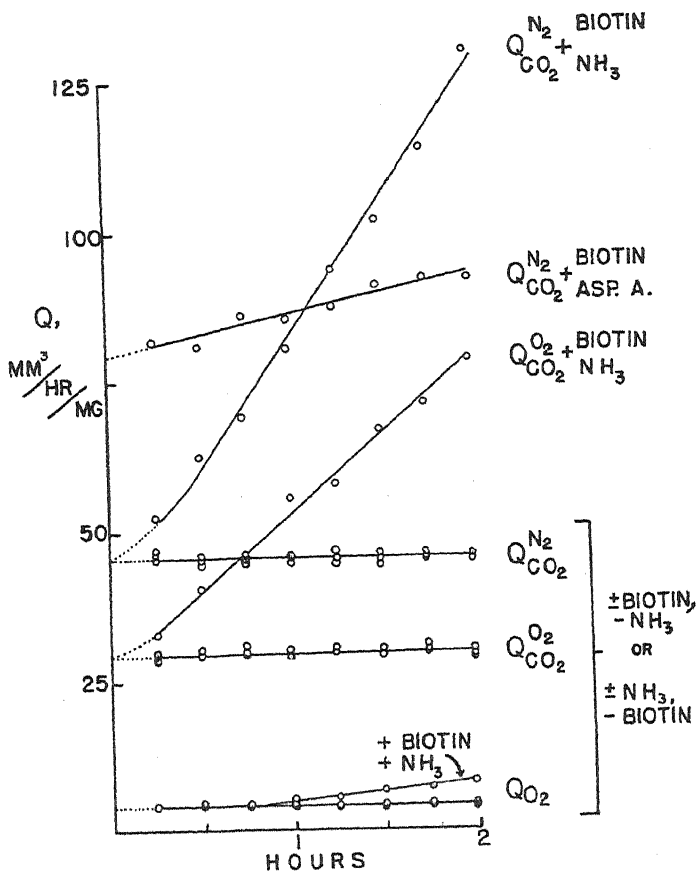


FIG. 1

The Effect of Biotin, Ammonia, and Aspartic Acid on the Respiration and Fermentation of Biotin-deficient Yeast

Yeast grown at $3.7 \times 10^{-6} \gamma$ biotin/cc. was washed and suspended in salt medium and 2% glucose. $7.4 \times 10^{-3} \gamma$ biotin, 100 γ nitrogen as ammonium sulfate, and 10 γ aspartic acid were added from side arms at zero time as indicated. Liquid volume 2 cc. Anaerobic experiments in nitrogen and aerobic experiments in air. Temperature 30°C. The symbols Q_{N_2} , Q_{CO_2} , and Q_{O_2} represent anaerobic fermentation, aerobic fermentation, and respiration, respectively, in mm.³/hr./mg. dry wt. "±" indicates "presence or absence."

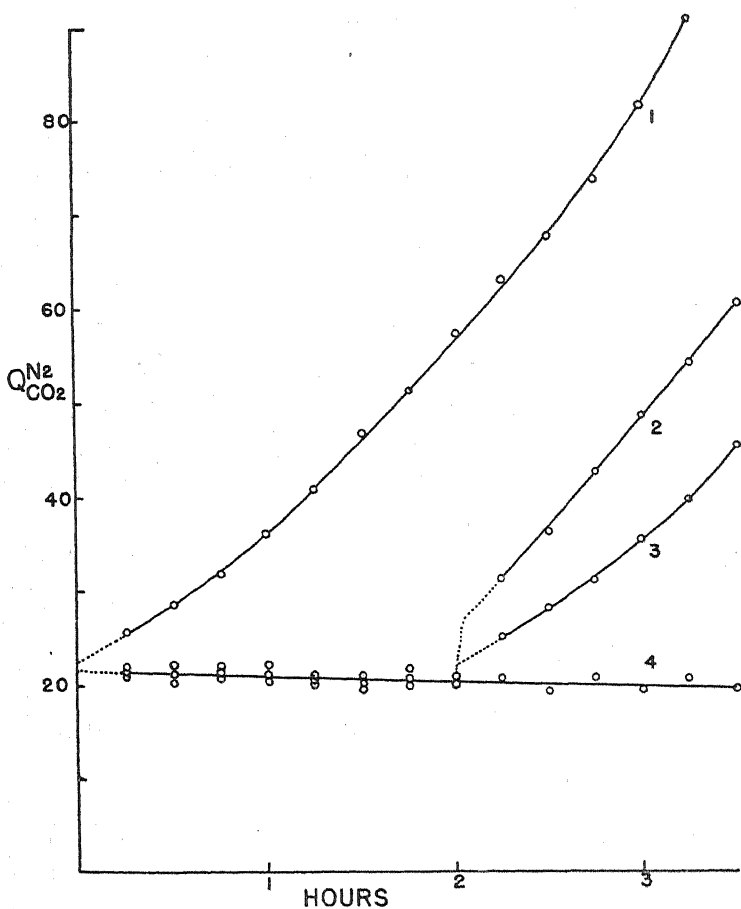


FIG. 2

The Effect of Biotin and Ammonia Pretreatment upon the Subsequent Anaerobic
Biotin Effect

Biotin-deficient yeast was washed and suspended in salt-glucose solution. Total liquid volume was 2 cc. Temperature 30° C. Nitrogen gas. 7.4×10^{-3} γ biotin and 200 γ ammonia nitrogen were added from side arms as follows:

- Curve 1. Biotin and ammonia added at $t=0$.
- Curve 2. Biotin at $t=0$ and ammonia at $t=2$ hrs.
- Curve 3. Ammonia at $t=0$ and biotin at $t=2$ hrs.
- Curve 4. No biotin and no ammonia added.

large initial stimulation of fermentation in normal yeast such as observed by Smythe (10, Fig. 3) and by many others in industrial yeast fermentations. It seems reasonable to suppose that the incubation of biotin-deficient yeast with biotin restores the ability of the yeast to respond normally to the addition of ammonia. Thus it would appear that the well-known immediate effect of ammonia on fermentation is in some way dependent upon the presence of adequate internal biotin.

Influence of other nitrogen compounds on the biotin effect

We were led to use other nitrogen sources in order to see if the action of ammonia in the *biotin effect* was a specific one. Several nitrogen compounds were added in anaerobic experiments with biotin-deficient yeast in the presence and absence of biotin, and the fermentation of glucose followed in the Warburg apparatus. The stimulation results are given in Table II. With the exception of aspartic acid, none of the nitrogenous compounds tested stimulated fermentation in the absence of biotin. In the presence of biotin, all compounds tested except leucine and glycine caused a gradual increase in fermentation rate, but none gave so marked an effect as ammonia. Since the action of the other compounds might be mediated through ammonia formed from them, it is possible that ammonia is highly specific for the biotin stimulation of metabolism although this cannot be definitely decided from the results. In any event, it is evident that leucine and glycine were not rapidly deaminated by biotin-deficient yeast or they, too, would presumably have caused an increase in the rate of fermentation under the influence of biotin.

Effect of aspartic acid

Of the nitrogenous compounds which appeared to substitute in some measure for ammonia, aspartic acid had a unique effect. At concentrations of 1 γ /cc. and above, aspartic acid caused a large and *immediate* stimulation of the rate of fermentation. This is shown in Fig. 1 as well as in Table II. The fermentation rates were usually increased by 75-100 per cent but there was little or no effect on oxygen consumption. Fig. 1 shows that while aspartic acid caused this large initial stimulation of fermentation, it served as a poor substitute for ammonia in giving the characteristic gradual increase with time under the influence of biotin. It will be shown in a later paragraph that fermentation of normal, biotin-rich yeast is also stimulated by aspartic acid. Citrate, fumarate, suc-

cinatc, glutamate, and asparagine did not cause this initial and immediate stimulation of fermentation in biotin-deficient yeast.

Experiments with other bios factors

We have thus far dealt with a yeast medium deficient primarily in biotin, the several other members of the B complex being present in the original growth medium. It was desirable to see if the results obtained with biotin-deficient yeast could be duplicated by analogous

TABLE II
The Effect of Nitrogen Source on Anaerobic Biotin Effect

N source	$Q_{CO_2}^{N_2}$ after 3 hours	Per cent increase over initial $Q_{CO_2}^{N_2}$
Ammonia.....	39.7	278
Asparagine.....	28.0	167
Urea.....	26.6	144
Arginine.....	22.0	105
Glutamic acid.....	16.8	60
Ornithine.....	15.0	42
Aspartic acid.....	26.9*	28
Leucine.....	10.5	0
Glycine.....	10.5	0
No nitrogen.....	9.0	-14

Biotin deficient yeast grown at 3.7×10^{-6} γ biotin/cc. washed and suspended in glucose-salt solution. All nitrogen compounds added at 1 mg./cc., and biotin at 1.6×10^{-3} γ /cc., at zero time; all amino acids of the *l* configuration series. Initial $Q_{CO_2}^{N_2} = 10.5$ except with aspartic acid = 20.9. Experiments in nitrogen gas at 30°C.

* Part of this rate is due to the specific and immediate effect of aspartic acid on $Q_{CO_2}^{N_2}$ and is not connected with the biotin effect involving time (see text). Initial $Q_{CO_2}^{N_2} = 21$.

experiments with deficiencies in others of these factors, in order to ascertain in how far the results with biotin are specific. Corresponding experiments were therefore performed in biotin-rich media, by leaving out individually others of the bios complex, and then adding the omitted factor in Warburg experiments similar to those described with biotin. It was found that our strain of yeast could grow practically as well without as with added thiamine, pyridoxin, and inositol, when each of these was left out separately. Likewise adding these factors to yeast grown in their respective absences had little or no effect upon the rate of fermentation

or upon the separate, usual increase with time obtained upon addition of ammonia to normal yeast. However, if β -alanine was omitted from the growth medium, no growth occurred. Yeast grown at low β -alanine concentrations had low rates of metabolism, but it was found that these did not increase until more than three hours after the addition of β -alanine or of pantothenic acid, either in the presence or absence of ammonia. Omission of β -alanine thus produced results most nearly resembling omission of biotin without yielding under our conditions, however, the full equivalent of a *biotin effect*.

The effect of azide

It has long been known that anaerobic fermentation is not affected by the usual concentrations of the respiratory poisons carbon monoxide, cyanide, and azide. However, it has been shown that these substances may affect carbohydrate synthesis at concentrations even lower than those inhibiting respiration (11, 12, 13). If this inhibition of synthesis by respiratory poisons is a general phenomenon, the possibility arises of determining whether the increase in metabolism elicited by biotin is the result of synthetic reactions resulting in the formation of certain substances which are concerned in the fermentation chain of reactions. If this is the case, it might be expected that addition of azide or cyanide would not affect the given initial rate of anaerobic fermentation, but would prevent occurrence of the *biotin effect*. This indeed proved to be the case, as is shown by Fig. 3. At 10^{-5} *M* azide there was a slight *biotin effect*, but at 10^{-4} *M* and 10^{-3} *M* this was entirely absent. In no case was there inhibition of the initial rate of fermentation by the azide. It was also found that ammonia uptake was completely inhibited by the two higher azide concentrations.

It thus appears that certain synthetic reactions involving nitrogen assimilation may accompany or precede the increase in fermentation rate which is caused by the combined action of biotin and ammonia. These synthetic reactions are blocked by low concentrations of azide (or of cyanide). Details of these initial azide-sensitive steps are still obscure, but it is possible that the mechanism of biotin action will be elucidated when their nature is known.

Normal- and biotin-intermediate yeast

Thus far the data have been mainly concerned with biotin-deficient yeast, *i.e.*, yeast grown at biotin concentrations of about 3×10^{-6} γ /cc.

and giving approximately one-fifth of maximum growth. Experiments identical with those shown in Fig. 1 were performed with yeast grown for 20 hours at 30° C. at a high biotin level (3×10^{-2} γ /cc.). Typical

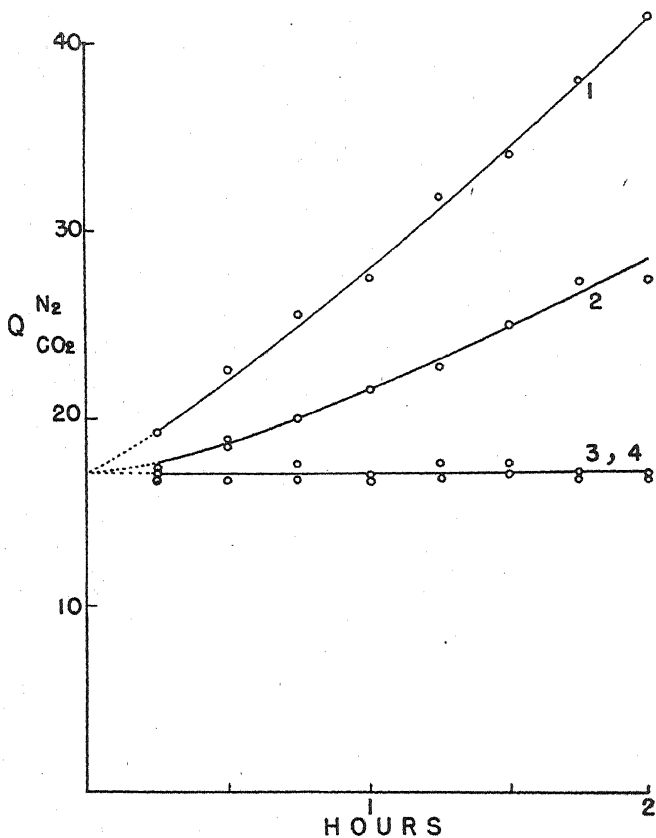


FIG. 3.

Inhibition of the *Biotin Effect* by Sodium Azide

Biotin-deficient yeast was washed and suspended in salt-glucose solution, pH 4.2. 100 γ ammonia nitrogen, 7.4×10^{-3} γ biotin, and sodium azide were added at zero time. Warburg experiments at 30° C. in nitrogen with 2 cc. liquid. Curves 1, 2, 3, and 4 were with 0, $10^{-5}M$, $10^{-4}M$, and $10^{-3}M$ sodium azide respectively.

results are shown in Fig. 4. In no case was there an increase in turbidity of more than 5 per cent in 2 hours after the addition of biotin and ammonia. The "normal" yeast of Fig. 4 had rates of respiration and

fermentation about ten times as high as the biotin-deficient yeast of Fig. 1, as is also indicated in Table III. These rates increased still more markedly in the presence of ammonia, but the addition of biotin had no

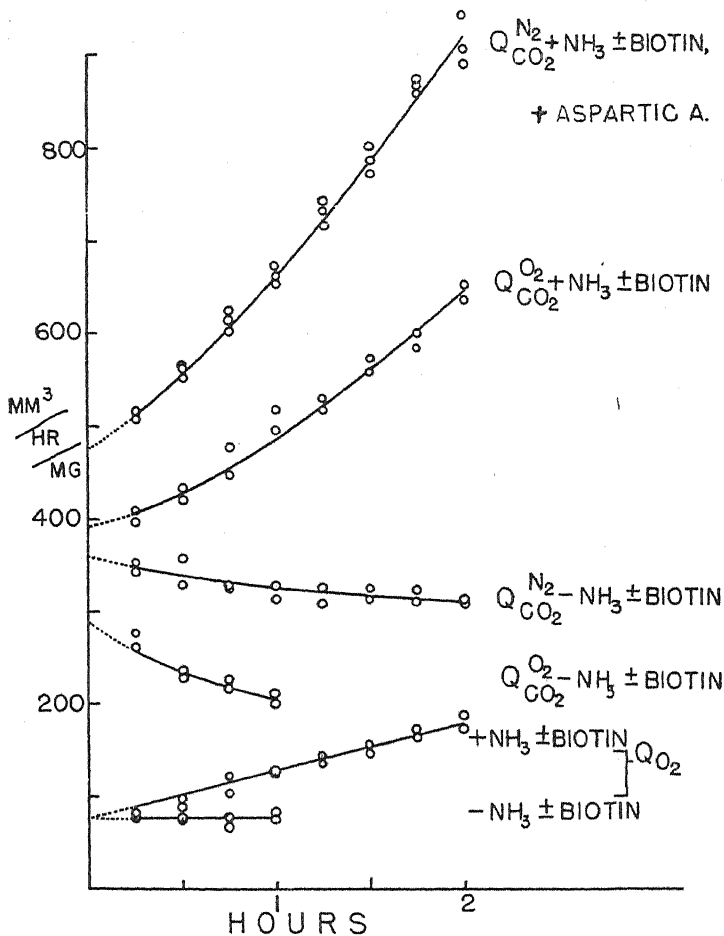


FIG. 4

The Effect of Biotin, Ammonia, and Aspartic Acid on Normal Yeast
Yeast grown at a high biotin concentration ($0.03 \gamma/\text{cc.}$) was washed and suspended in salt-glucose solution. At zero time $7.4 \times 10^{-3} \gamma$ biotin, 200γ ammonia nitrogen, and 100γ aspartic acid were added from side arms as indicated. Symbols, temperature and liquid volume as in Fig. 1.

influence upon this increase. The latter result was to be expected since the yeast presumably already had sufficient biotin for its needs. The addition of either aspartic acid or ammonia caused an initial fermentation stimulation, followed by a more gradual increase.

The results of a typical experiment with "biotin-intermediate" yeast (grown at 3×10^{-5} γ biotin/cc. and giving almost half-maximum growth) are shown in Fig. 5. The metabolic behavior is intermediate between that of the biotin-deficient and the normal yeasts. The rates of fermentation, which are intermediate (Table III), tend to fall off rapidly

TABLE III
Metabolic Values of Yeasts Poor, Intermediate, and Rich in Biotin

Yeast	Exp. No.	I Q _{O₂}	II Q _{CO₂}	III Q _{N₂} Q _{CO₂}	Pasteur effect			
					Abs.	%	M.O.Q.	
					III - II	(III - II)100	III - II	
					III	I/3		
Biotin deficient	I	a	9.3	46	74	28	38	9.0
		b	15.0	165	213	48	22	9.6
	V	a	3.7	50	62	12	20	10.0
		b	12.5	95	175	80	46	19.2
	XVII	a	2.8	45	56	12	21	12.6
		b	6.2	70	85	15	18	7.4
	XXXI	a	4.2	30	47	17	36	12.1
		b	7.7	77	123	45	37	17.7
Biotin intermediate	XXX	a	14.0	99	129	30	23	6.4
		b	24.9	333	430	97	23	11.7
Biotin-rich (normal)	XVII	a	70	280	350	70	20	3.0
		b	180	645	900	255	28	4.2
	XIII	a	75	186	278	92	33	3.7
		b	127	357	550	193	35	4.6

Under each experiment the first row marked by *a* is in the absence of biotin and ammonia. The second horizontal column marked *b* is for the same yeast after two hours in the presence of biotin and ammonia.

with time unless biotin and ammonia are present. The fall in fermentation rate was often very rapid, the rates decreasing perhaps to one-half in two hours. The presence of ammonia alone (without biotin), or of oxygen, usually accelerated the drop, but the presence of biotin (without ammonia) tended to prevent it. Addition of both biotin and ammonia brought forth a rapid increase in fermentation and respiration rates. In this case the respiration increase did not lag as much behind the fermentation increase as in biotin-deficient yeast. There was a small initial stimulation by ammonia, and a small aspartic acid stimulation,

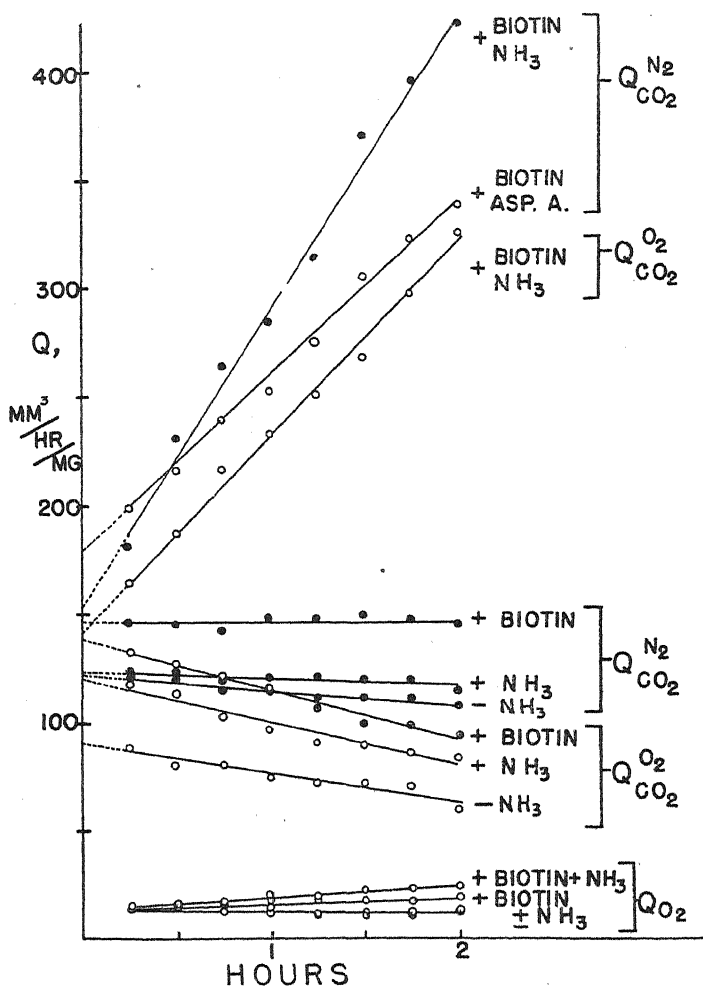


FIG. 5

The Effect of Biotin, Ammonia, and Aspartic Acid on the Metabolism of Biotin-intermediate Yeast

Yeast grown at a biotin concentration of $3 \times 10^{-5} \gamma$ biotin/cc. for 20 hours was washed and suspended in salt-glucose solution, $7.4 \times 10^{-3} \gamma$ biotin, 100γ ammonia nitrogen, and 100γ aspartic acid were added at zero time as indicated. Anaerobic experiments in nitrogen and aerobic experiments in air. Temperature 30°C .

and ammonia gave a much greater increase with time than did aspartic acid. A most important observation was a definite *immediate* increase of fermentation caused by biotin, with or without added ammonia. This immediate increase was usually, but not always, observed in many cases of biotin-intermediate yeast tested, and was not measurably a function of time. As with the biotin-deficient yeast, there was none of the usual *biotin effect* unless both biotin and ammonia (or certain other nitrogenous compounds) were present.

The biotin-intermediate yeast was very variable, and not all the characteristics in Fig. 5 always appeared in any given preparation. Such factors as the amount and condition of the inoculum, length of incubation, etc. have to be carefully controlled in order to obtain yeast intermediate in all respects simultaneously.

Biotin balance in yeast

In order to determine whether our yeast synthesized or destroyed biotin, as well as to determine the possible variation in intra-cellular biotin content, a series of biotin-balance experiments were performed at four different levels of biotin. At each biotin level three flasks containing 30 cc. of the growth medium were inoculated with 0.06 mg. dry weight of fresh yeast. These four groups of flasks were inoculated with 1 γ , 0.01 γ , 0.001 γ , and 0.0001 γ of crystalline biotin respectively. After 19 hours at 30° C., the yeast dry weight of the first flask of each series was determined by the turbidimetric method. Each was then centrifuged and the biotin content of the supernatant and of the hydrolyzed yeast was determined. The total nitrogen content of the yeast was measured by the micro-Kjeldahl method. At 27 hours the procedure was repeated with the second flask of each series and at 38 hours with the last flask. In another experiment 0.0001 γ of biotin was added to a flask similarly set up and at 24 hours the dry weight and the biotin in the yeast and in the medium was determined. The results are given in Table IV. In all cases there was, within the experimental error, complete recovery of the added biotin, indicating that neither synthesis nor destruction of biotin had occurred.

Table IV shows that the biotin content ranged from 0.017 γ biotin/g. dry weight yeast to 13.9 γ /g., an eight hundred fold difference. The yeast used in this work contained about 3.5×10^{10} cells/g. dry weight. The biotin content of the yeast of Table IV thus lies between the limits of one thousand and one million molecules per cell.

The uptake of biotin and ammonia by biotin-deficient yeast

The foregoing biotin balance studies were extended to kinetic aspects of the uptake of both biotin and ammonia by biotin-deficient yeast, and the effect of various conditions upon this uptake. The experiments were carried out with biotin-deficient yeast grown and washed as before. It was shaken under anaerobic conditions in Warburg vessels at 30° C., biotin and ammonia being added from vessel side arms after temperature equilibration. After two hours the suspensions were poured from the vessels, centrifuged, and the supernatant analyzed for biotin and ammonia. Control Warburg experiments were made in which the rates of

TABLE IV

Biotin Balance: Recovery of Nutrient Biotin in Yeast Crop

Concen- tration of biotin added	Hrs. after inocu- lation	Add- ed	γ Biotin/30 cc. Recovered			Mg. dry wt. of nitrogen yeast (final)	Mg. γ Biotin/ in yeast (final)	Per cent dry wt. yeast	Per cent nitro- gen
			Yeast	Medi- um	Total				
I. Very high	19	1.0	0.27	0.67	0.94	19.2	2.2	13.9	11.5
	27	1.0	0.27	0.67	0.94	39.6	3.78	6.7	9.6
	38	1.0	0.27	0.67	0.94	47.4	3.65	5.6	7.7
II. High	19	0.01	0.011	0	0.011	15.0	2.06	0.73	13.7
	27	0.01	0.011	0	0.011	34.2	3.34	0.32	9.8
	38	0.01	0.011	0	0.011	42.0	3.65	0.24	8.8
III. Inter- medi- ate	19	0.001	0.001	0	0.001	7.2	0.88	0.14	12.3
	27	0.001	0.001	0	0.001	14.4	1.48	0.7	10.3
	38	0.001	0.001	0	0.001	15.6	1.48	0.6	9.5
IV. Very low	19	0.0001	0.0001	0	0.0001	6.7		0.017	

fermentation and the turbidities under the various conditions were simultaneously determined. The results of a typical experiment are given in Table V.

The maximum assimilation of both biotin and ammonia is shown in Column 1, Table V. Here the biotin uptake amounted to 12.9 γ /g. of yeast. By reference to Table IV for comparison, it becomes apparent that the biotin-deficient yeast in Table V had regained its normal complement of biotin, *i.e.*, had become "biotin-rich," within two hours. This surprisingly rapid recovery in biotin content was accompanied by only a partial recovery in metabolic activity. The maximum ammonia uptake (Column 1) was 40 mg. N/g. of yeast (4 per cent). Since from

Table IV the normal nitrogen content of our yeast ranges around 10 per cent it is evident that the total nitrogen increased by about 40 per cent in two hours. This was not accompanied by any significant growth (increase in turbidity).

Column 2, Table V, confirms our earlier conclusion that no ammonia is used by biotin-poor yeast in the absence of added biotin. It is to be

TABLE V
Factors Affecting Biotin and Ammonia Uptake

Addition	Concentration	Medium					
		1	2	3	4	5	6
		Complete	Minus biotin	Minus ammonia	Minus glucose	Minus phosphate	Plus azide
KH ₂ PO ₄	2 mg./cc.	+	+	+	+	—	+
Glucose	20 mg./cc.	+	+	+	—	+	+
NH ₃ -nitrogen	150 γ	+	+	—	+	+	+
Biotin	0.037 γ	+	—	+	+	+	+
Azide	10 ⁻³ M	—	—	—	—	—	+
Biotin-deficient yeast	γ Biotin taken up	0.029	—	0.021	0.007	0.012	—
	γ nitrogen taken up	90	0	—	0	20	0
	Initial Q _{CO₂} ^{N₂}	44	44	44.0	0	36	42
	Final Q _{CO₂} ^{N₂}	108	44	44.0	0	36	42
Normal yeast	γ ammonia taken up	70	70	—	0	20	0
	Initial Q _{CO₂} ^{N₂}	337	337	—	0	247	337
	Final Q _{CO₂} ^{N₂}	873	864	—	0	247	337

Biotin-deficient yeast grown at 3.7×10^{-6} γ biotin/cc. washed and suspended in distilled water. 2.25 mg. dry wt. yeast used in each vessel. All additions were made to a final volume of 2 cc. at zero time. After following the fermentation for 2.5 hrs., the suspension was analyzed for biotin and ammonia. The normal yeast was grown at 0.1 γ biotin/cc., washed by centrifugation, and suspended in distilled water; 0.73 mg. dry wt. yeast used per vessel.

noted from Column 3, however, that the converse is not true; almost as much biotin is taken up in the absence of ammonia as in its presence. There was, however, no increase in the rate of fermentation in the absence of ammonia. That biotin could be taken up in the absence of ammonia was indicated in the "pretreatment" experiment already described. There, the incubation of biotin-deficient yeast with biotin had no in-

fluence on the rate of fermentation. When ammonia was added to such pretreated yeast, however, the increase in fermentation rate was much more marked than when biotin and ammonia were added together. The dependence of ammonia assimilation on the presence of biotin makes it probable that, in inadequate biotin, growth ceases in part because of the inability of the yeast to assimilate nitrogen.

In the absence of glucose (Column 4) the rate of fermentation was nearly zero, of course, and the biotin-deficient yeast took up little or no ammonia and relatively little biotin. This interesting result was forecast by metabolism experiments in which biotin-deficient yeast was incubated anaerobically in the presence of both biotin and ammonia, but without substrate. After a two hour incubation period, glucose was added and the rate of fermentation followed. The results were not different from those in a parallel experiment in which all three substances were added at the same time, indicating that the biotin and ammonia had had no effect on the yeast in the absence of glucose. Thus it appears that the assimilation of ammonia and probably of biotin are active processes requiring energy or materials made available in metabolism.

The latter possibility has already been considered in regard to ammonia utilization, where it has been shown that azide inhibits the biotin stimulation effect without decreasing the initial rate of fermentation. In Column 6, Table V, the uptake of ammonia is shown to be completely inhibited by 10^{-3} *M* sodium azide. The initial rate of fermentation, however, was entirely unaffected. The inhibition of ammonia uptake by azide is further evidence that the assimilation requires the participation of metabolism. The azide sensitivity indicates that heavy metals are involved in ammonia assimilation, even if only indirectly.

In Column 5, Table V, the solution was complete except for the lack of phosphate, and here there was markedly diminished biotin and ammonia utilization. With lack of phosphate there was a definite lowering of fermentation rate, and it is difficult to say whether the inhibition of ammonia and biotin assimilation is specifically due to the lack of phosphate or to the effect on metabolism.

The uptake of ammonia by normal yeast

The experiment just described has shown that ammonia assimilation by biotin-deficient yeast is almost completely inhibited by lack of biotin, glucose, or phosphate, and by the presence of azide. The uptake of ammonia by normal yeast and the influence of the above conditions upon

this uptake and upon fermentation is also given in Table V. The experiments were performed under conditions identical with those given above for biotin-deficient yeast. It is clear that lack of substrate and phosphate and the presence of azide again inhibited the assimilation of ammonia. The presence of biotin, as is to be expected, had no effect on ammonia uptake under these conditions. The rate of fermentation was again slightly inhibited by lack of phosphate and was unaffected by azide. The increase in fermentation rate observed in Columns 1 and 2 was prevented by azide and by lack of phosphate. The similarities between biotin-deficient and normal yeast here make it probable that nitrogen assimilation is easily interfered with by conditions affecting metabolism, as is also true of biotin uptake.

DISCUSSION

Since neither biotin nor ammonia alone had any observed effect on biotin-deficient yeast (except biotin in the "pretreatment" experiments), whereas together they caused a marked, gradual rise in rate of metabolism it appears quite probable that there is involved a synthesis of nitrogenous material that is ultimately or cyclicly connected with the metabolism of glucose. It is unlikely that this could be merely a compound formed directly between biotin and ammonia nitrogen because more than a million molecules of ammonia nitrogen for each biotin molecule are necessary to give maximum effects. It is quite possible that biotin in some way brings about the assimilation of ammonia nitrogen which then results in the synthesis of certain materials or enzymes involved in the fermentation system, and thus increases the rate of fermentation, and in turn respiration and growth. However, the fact that biotin can cause, in biotin-intermediate yeast, an immediate stimulation of fermentation in the absence of ammonia is indication that biotin itself may well participate directly in the fermentative system. Moreover, the need for active metabolism *per se* for the assimilation of ammonia makes it difficult to say whether biotin is more directly concerned with nitrogen assimilation or with fermentation in the cycles involving both these intimately related processes. It has not yet been feasible to make a clear-cut temporal separation of these two processes, as was possible between fermentation, respiration, and growth.

The experiments have certain implications bearing on the nature of the changes occurring in the preliminary portions of the normal growth curve of microorganisms generally. It is well-known that when inocula

of most microorganisms are seeded into a new medium there occurs an initial period in which there is no growth, often called the initial stationary period or *pre-lag* phase. Our experiments have shown that the cells of biotin-deficient yeast do not immediately start dividing upon inoculation into a new medium, but first pass through an abnormally prolonged phase where the rate of metabolism increases, ammonia is utilized, etc. It is quite evident that in such yeast biotin is playing a very important role in initiating the changes in metabolism which lead to growth during the lag and logarithmic growth phases.

Another noteworthy observation derivable from the data is the uniquely high Meyerhof oxidation quotient (M.O.Q.) of 10 to 20 given by biotin-deficient yeast (Table III). This quotient

$$3(Q_{\text{CO}_2}^{\text{N}_2} - Q_{\text{CO}_2}^{\text{O}_2})/Q_{\text{O}_2}$$

is one numerical characterization of the Pasteur effect, and expresses the magnitude of the aerobically induced decrease in fermentation in relation to concurrent oxygen consumption. Experimentally it has been found that essentially all living, intact, fermenting, and glycolyzing tissues thus far examined have given quotients of about 3-6. Burk has recently reviewed the subject (14, 15), and has shown that Meyerhof oxidation quotients above about 10 are, in any event, thermodynamically impossible in terms of the resynthesis of fermentation products to higher carbohydrates.

Biotin-deficient yeast provides, essentially for the first time with intact cells, Meyerhof oxidation quotients which lie consistently between 10 and 20. This is brought out in Table III, where the results of several experiments with biotin-deficient, biotin-intermediate, and normal yeasts are summarized. These high quotients above 10 constitute strong evidence for the view that not aerobic resynthesis but some other result of the presence of oxygen is the factor responsible for the aerobic decrease in aerobic fermentation rate compared to anaerobic fermentation. This effect could well be brought about through the maintenance of some fermentation catalyst in an oxidized or inactive state, as originally proposed by Lipmann (16) and elaborated with respect to details by Ball (17) and many others since (15, Table I) Lipmann reported that high Meyerhof oxidation quotients of 10 to 20 could be obtained in cell-free extracts, and has suggested that the Pasteur effect there is due to the inhibition of fermentation as a result of some catalyst in the fermentation system being maintained in an oxidized, inactive form. Thus, the

measured net fermentation would be decreased aerobically, not because of the resynthesis of carbohydrate from the fermentation products, but simply because the latter never formed.

SUMMARY

1. Biotin-deficient yeast was found to respire and ferment at rates one tenth to one twentieth of those of normal biotin-rich yeast. Upon the addition of biotin, in the presence of ammonia, but not in its absence, these metabolic rates rise gradually, fermentation first, then respiration, and finally growth. With yeast only partly deficient in biotin, added biotin may cause an immediate increase in fermentation rate, even without added ammonia, but this increase is not a function of time. The yeast studied was *Saccharomyces cerevisiae*, Fleischmann strain 139.

2. Other nitrogen sources, particularly asparagine, arginine, and urea, may substitute for ammonia but are far less effective. Leucine and glycine cannot substitute for ammonia in its effect on the action of biotin on metabolism.

3. Low concentrations of azide and cyanide can, without affecting the initial fermentation rate, prevent the increase in the rate of fermentation elicited by biotin and ammonia. Azide also inhibits the utilization of ammonia by yeast.

4. The effects described were not duplicated by adding other growth factors to yeast grown in their respective absences, although yeast grown at inadequate levels of β -alanine (a pantothenic acid vitamer) were observed to have very low rates of metabolism.

5. Biotin was neither synthesized nor destroyed by growing yeast. The biotin content of yeast, when grown at different biotin levels varied from 0.017 γ to 13.9 γ /g.

6. Biotin-deficient yeast rapidly takes up from solution its normal complement of biotin. This biotin uptake is markedly decreased in the absence of glucose or phosphate, and appears to involve synthetic processes.

7. No ammonia was taken up by biotin-deficient yeast unless biotin was present. Ammonia uptake by normal and biotin-deficient yeast was almost completely inhibited by lack of glucose or phosphate, and by the presence of 10^{-4} *M* azide.

8. The Meyerhof oxidation quotients of biotin-deficient yeast range from 10 to 20 compared to usual values of 3 to 6 or less. The significance of this in relation to the Pasteur effect is discussed.

One of us (R. J. W., now Assistant Professor of Biochemistry, University of Southern California School of Medicine, Los Angeles) wishes to acknowledge the tenure of a National Research Council Fellowship in the Medical Sciences during the main course of this work (1940-41).

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An Improved Phenolphthalin Technique for the Micro-Determination of Cyanide

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INTRODUCTION

Studies of the effect of cyanide on the oxygen consumption of tissues and enzyme systems may involve the use of hydrocyanic acid solutions that are too dilute to permit a measurement of the cyanide content by the ordinary quantitative techniques. Since HCN is volatile and its solubility varies with differing conditions of temperature and osmotic equilibrium, it is sometimes desirable to make a direct check of its concentration during an experiment. With the method presented below it is possible to determine quantitatively as low a concentration of cyanide as one-one hundred thousandth molar in only two milliliters of fluid.

The original publication of the phenolphthalin method by Weehuizen in 1905 (1) gives only a brief statement of the principle of the reaction, but several later investigators (2, 3, 4, 5) have presented more detailed techniques. None of these workers claim to obtain more than roughly quantitative results; the amounts of fluid required for the test are large, and both the reagent used and the color produced are unstable. The experiments described below show how some of these difficulties were overcome. The advantages of the present method over those previously described are the reproducibly quantitative technique and the simplicity and rapidity of the determination.

EXPERIMENTAL

The reaction depends upon the oxidation of phenolphthalin to phenolphthalein by cyanide and copper. The molar concentrations of the reacting substances (calculated from the data shown in Fig. 1), indicate that the phenolphthalin oxidation is probably the result of the reduction

of copper by cyanide to form cuprous cyanide. Addition of alkali converts the phenolphthalein thus formed to the red di-potassium salt, and the cyanide concentration may then be measured by determining the amount of color in the solution.

Preparation of Reagent

Phenolphthalin is insoluble in water, slightly soluble in dilute alkali, and soluble in alcohol. Alkali tends to cause the substance to shift to

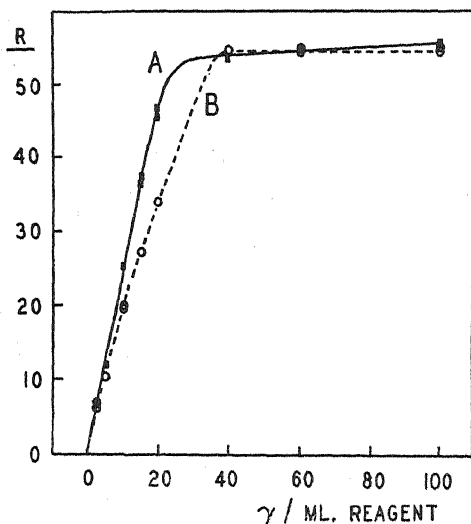


FIG. 1

Quantities of Phenolphthalin and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ Necessary for Development of Color of 0.00015 *M* KCN Solution

Abscissa indicates γ chemical /ml. reagent; ordinates for all figures represent readings on logarithmic scale of electrophotometer. For further details see text.

the phenolphthalein form and alkaline solutions are therefore unstable. If the chemical is first dissolved in ethanol a small amount of the solution may be added to water without any precipitation occurring. This quantity is enough to allow a complete cyanide reaction. The phenolphthalin in the resulting mixture is much more stable than in one which contains alkali.

To determine the minimum quantity of the phenolphthalin that still would give the maximum color with the highest concentrations of cyanide

used, the experiment represented by Curve A in Fig. 1 was made. A series of aqueous solutions was prepared from 0.1% phenolphthalin in absolute ethanol in such a way that the final concentrations varied from zero to 100 γ per ml. Enough $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was added to each to make 50 γ per ml. of this material. The effectiveness of these solutions in giving a reaction with 0.00015 *M* KCN was then determined by adding 1 ml. of each to 2 ml. of KCN plus 1 ml. of 0.05% KOH. The ordinates of Fig. 1 give the readings obtained on the logarithmic scale of the electrophotometer¹; the abscissas show the phenolphthalin concentrations of the reagents in γ /ml. Since amounts above 40 γ /ml. show little change in the electrophotometer readings, a quantity of 50 γ /ml. was considered to be sufficient for a standard reagent.

The optimum amount of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was found similarly by varying the amount of this chemical and keeping the phenolphthalin at 50 γ /ml. Curve B in Fig. 1 shows that with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ also there is little change after the 40 γ /ml. point has been reached. The slope of the curve is less steep than with the phenolphthalin and to be certain of an excess the standard reagent was made up to contain 100 γ /ml. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

To determine the optimum amount of alkali, 0.025 to 1.0 ml. quantities of 1% KOH were placed in microcolorimeter tubes and sufficient water was added to make the total volumes 1 ml. each. Two ml. portions of the cyanide solutions, the concentrations of which are indicated on the graph (Fig. 2), and 1 ml. of a reagent containing 50 γ of phenolphthalin and 100 γ of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per ml. were then added and the amounts of color determined. The curves show that for all three cyanide concentrations 0.05 ml. or 1% KOH of 500 γ is sufficient to produce the maximum amount of color. In subsequent experiments this optimum amount of alkali was added by using 1 ml. of 0.05% KOH solution. If more alkali is added there is a progressive decrease in intensity. When the alkali concentration is increased as much as 20 times the optimum amount, the color produced is actually decreased 25%; moreover, the red color fades as the solution stands.

The amount of alkali which gives the maximum color is, as may be seen from Fig. 2, very close to the sharply descending portion of the curve. A slight increase in acidity may therefore cause the color to decrease or disappear. If the cyanide solutions to be tested are not neutral or

¹ A Fisher Electrophotometer was used for all of the determinations recorded in the present paper.

slightly alkaline it may be necessary to adjust the amount of KOH accordingly in order to secure the optimum reading.

Effect of Excess Phenolphthalin

In addition to the primary cyanide reaction there is a slow spontaneous conversion of the phenolphthalin which causes an increase in the red color as the test solution stands. The following experiments show some-

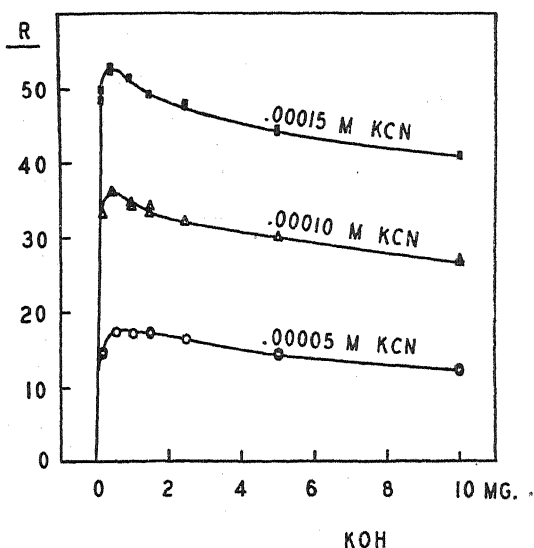


FIG. 2

Variation with Alkali Concentration of Amount of Color Produced in Phenolphthalin Reaction

thing of the nature of this secondary reaction and indicate how possible interference from it may be reduced to a minimum.

Curve A in Fig. 3 indicates the change that took place in the amount of color produced by a 0.0001 M KCN solution during the ten minute period after the cyanide, alkali, and reagent were mixed. For this curve the optimum amounts of KOH, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and phenolphthalin were used, that is, 500 γ , 100 γ , 50 γ respectively. The amount of color observed at ten minutes was about 3% more than that at one minute after mixing. With an excess of phenolphthalin, however, there is a considerable increase in the amount of color that develops after the orig-

inal cyanide reaction. Curve B shows the change when 400 γ of phenolphthalin are used in the reagent for 2 ml. of 0.0001 M KCN (3) instead of 50 γ . In this case there is a 17% increase in color during the ten minute interval. The additional color is apparently due to the conversion to phenolphthalein of some of the phenolphthalin remaining after the cyanide reaction. Curve C shows that if only 10 γ of phenolphthalin are used for 2 ml. of 0.0001 M KCN there is no change in the amount of color during the ten minute period. This quantity is less than that re-

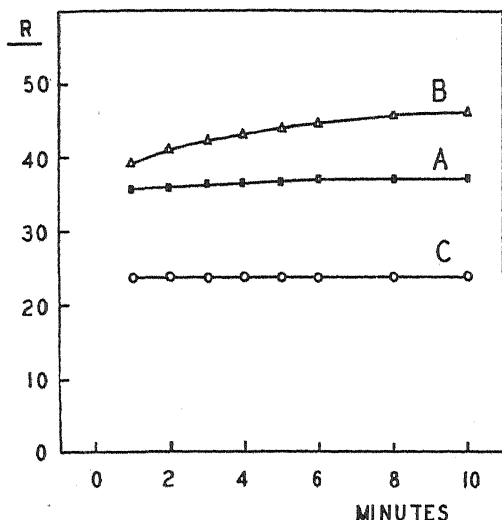


FIG. 3

Change during the Ten Minute Period Following the addition of Reagent to Cyanide and Alkali

- (A) Control.
- (B) Excess phenolphthalin.
- (C) Insufficient phenolphthalin.

quired for a complete reaction and presumably all of the substance is changed immediately to the phenolphthalein form. It is apparent that most of the troublesome spontaneous change in color which may follow the immediate cyanide reaction is eliminated by reducing the phenolphthalin concentration in the reagent to a minimum that still will give a complete reaction.

Influence of Oxygen. Some investigators have suggested that solu-

tions used in the test should be made up in boiled water so that spontaneous oxidation of the phenolphthalin by dissolved oxygen may be avoided (3). When there is not an excess of phenolphthalin in the reagent this precaution is apparently unnecessary. If a stream of oxygen is bubbled through a solution of 0.0001 *M* cyanide with optimum concentrations of alkali and reagent, the color curve for a ten minute period is almost identical with that of the control (Curve A). Even if the bubbling is continued for an hour period there is only a slight increase when compared to a control tube left standing in air.

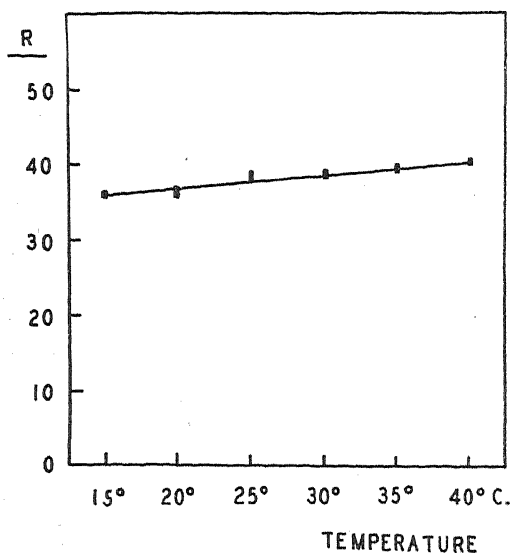


FIG. 4

Amount of Color Produced by Phenolphthalin Reaction with 0.0001 *M* KCN over a Temperature Range of 15°C. to 40°C.

Influence of Temperature. One of the phenolphthalin methods for cyanide determination (2), states that the test should be run at a temperature of 15° C. If there is excess of phenolphthalin in the reagent a slight change in temperature may cause considerable variation in the amounts of color which develop. With the improved reagent, however, this variation is reduced to a minimum. Fig. 4 shows that even though the temperature at which the reaction takes place is raised from 15° C. to 40° C., the amount of color produced increases only about 10%. If

a standard concentration curve for cyanide is made at a temperature of 25° C. and at the time an unknown solution is determined the room temperature happens to be as low as 20° or as high as 30° C., the maximum error involved due to the temperature difference is only about 2.5%.

The Concentration Curve

Fig. 5 shows a curve obtained by plotting the electrophotometer readings of KCN solutions of 0.00001 *M* to 0.00015 *M* when treated with alkali and reagent as outlined. The curve is regular and nearly a straight line. For seven concentrations along this curve the maximum deviations within groups of ten identical samples were less than 3%.

The sensitivity of the determination is such that if the 2 ml. sample analyzed contains 0.5γ of CN' (1 part in 4,000,000), the maximum deviation expected is still within the 3% limit. One half of this quantity of cyanide, that is, one part in eight million, will still give a positive test.

Specificity

The phenolphthalin test is not entirely specific for cyanide: ferri-cyanides and aqueous solutions of halogens give a similar color (2), because of direct oxidation of the phenolphthalin. Interference may come from sulfides, which precipitate copper from the reagent, or from phenol (2), or ferrocyanides (6) that prevent full color development. Among those substances which are reported as not interfering with the reaction are halogen salts, H_2O_2 , ferric chloride, chromates, and HNO_3 (2). If there is uncertainty about the identity of the reacting compound the qualitative silver iodide test of Fox (7) may be employed. This is specific for cyanide and is nearly as sensitive as the phenolphthalin reaction.

METHOD

The standard procedure for the micro-determination of cyanide resulting from the above experiments is outlined below.

To prepare the reagent, 0.5 ml. of 1% phenolphthalin (Eastman Kodak Company) in absolute ethanol is added to 99.5 ml. of 0.01% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and the two are mixed. This solution is stable for a day or more at room temperature and will keep in a refrigerator for months without change.

One ml. of 0.05% KOH is placed in a micro cell of a photoelectric colorimeter, 2 ml. of cyanide solution are added from a volumetric pi-

pette, and then 1 ml. of the reagent is added. The contents are mixed by inverting the tube several times and the color is determined immediately in the electrophotometer, using a #548 narrow range filter. The amount of cyanide in the unknown is then found by interpolating the reading on the logarithmic scale of the electrophotometer into a standard curve obtained with known concentrations of cyanide determined under identical conditions (see Fig. 5).

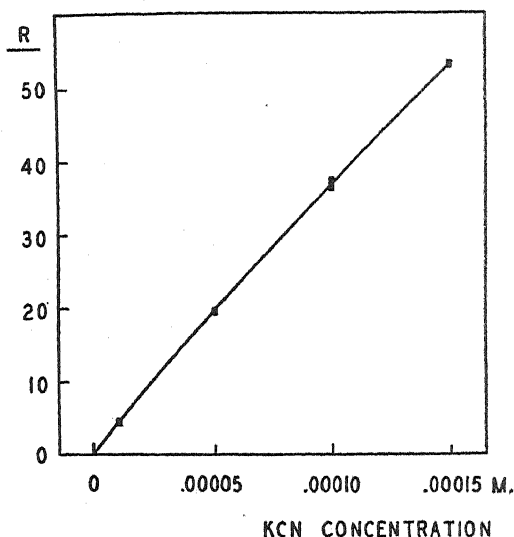


FIG. 5

Electrophotometer Readings for Cyanide Concentrations of 0.00001 *M* to 0.00015 *M* when Measured as Outlined in Text

If the cyanide is 0.00015 *M* or less the technique as outlined will give a color that is within the optimum range for photoelectric determination. Concentrations up to 0.01 *M* may be measured by diluting the cyanide solution one to ten or one to one-hundred with distilled water before taking the 2 ml. sample for analysis.

If larger quantities of the unknown solutions are available it is possible to make the test more sensitive by taking 12 ml. of cyanide instead of two, with 6 ml. each of alkali and reagent. Twenty-three ml. electrophotometer cells are used. The reading for a solution in a large cell is almost double that for the same solution in a smaller one.

The #548 filter transmits only a narrow band of light with a peak at 5480 Å. For electrophotometers that are not equipped with a narrow range filter in this zone the broad band type that is standard equipment on most instruments and has a peak at about 5250 Å. may be used. Readings with this filter are approximately two-thirds those obtained with the narrow transmission band.

Use of Buffered Solutions and Sea Water

In all of the experiments described above the KCN was dissolved in distilled water. If the liquid containing the cyanide to be tested is buffered it may be necessary to adjust the amount of KOH used. When 2 ml. of a 0.0001 *M* KCN solution in *M*/150 phosphate buffer (8) of pH 6.8 was tested, using 1 ml. of 0.05% KOH, the reading was only 2.2. When 0.1% KOH was used instead of the 0.05% KOH, the extra alkali was sufficient to overcome the buffering effect of the phosphate and a normal reading for this cyanide concentration resulted.

With sea water an excess of KOH must be avoided or the hydroxide precipitates of some of the salts may form. For 2 ml. of cyanide solution made up in artificial sea water (9) 1 ml. of 0.05% KOH caused no interference and gave the optimum color. When the alkali concentration was doubled precipitation occurred. There is somewhat less color formed, however, when the phenolphthalin reaction takes place in sea water. This amounts to about 15% with 0.0001 *M* KCN. To make concentration determinations it is necessary to prepare a reference curve with the standard cyanide solutions made up in sea water.

SUMMARY

A modified phenolphthalin method for the micro-determination of cyanide is presented with a critical analysis of its adequacy. 0.5γ of CN' or one part in four million is determinable within 3%. Only 2 ml. of cyanide solution are necessary. The reagent is made by adding 0.5 ml. of 1% phenolphthalin in absolute ethanol to 99.5 ml. of 0.01% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. This is stable at room temperature for a day or more and will keep in a refrigerator for months. Two parts of cyanide solution are added to one part of 0.05% KOH, one part of reagent is added, and the amount of red color is determined with a photoelectric colorimeter using a filter transmitting at about 5480 Å. The color changes only slightly with time and the variation in color with temperature is low.

slightly alkaline it may be necessary to adjust the amount of KOH accordingly in order to secure the optimum reading.

Effect of Excess Phenolphthalin

In addition to the primary cyanide reaction there is a slow spontaneous conversion of the phenolphthalin which causes an increase in the red color as the test solution stands. The following experiments show some-

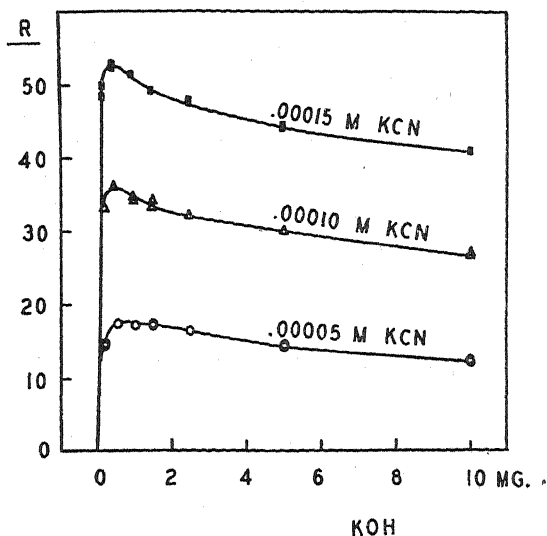


FIG. 2

Variation with Alkali Concentration of Amount of Color Produced in Phenolphthalin Reaction

thing of the nature of this secondary reaction and indicate how possible interference from it may be reduced to a minimum.

Curve A in Fig. 3 indicates the change that took place in the amount of color produced by a 0.0001 M KCN solution during the ten minute period after the cyanide, alkali, and reagent were mixed. For this curve the optimum amounts of KOH, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and phenolphthalin were used, that is, 500 γ , 100 γ , 50 γ respectively. The amount of color observed at ten minutes was about 3% more than that at one minute after mixing. With an excess of phenolphthalin, however, there is a considerable increase in the amount of color that develops after the orig-

inal cyanide reaction. Curve B shows the change when 400 γ of phenolphthalin are used in the reagent for 2 ml. of 0.0001 *M* KCN (3) instead of 50 γ . In this case there is a 17% increase in color during the ten minute interval. The additional color is apparently due to the conversion to phenolphthalein of some of the phenolphthalin remaining after the cyanide reaction. Curve C shows that if only 10 γ of phenolphthalin are used for 2 ml. of 0.0001 *M* KCN there is no change in the amount of color during the ten minute period. This quantity is less than that re-

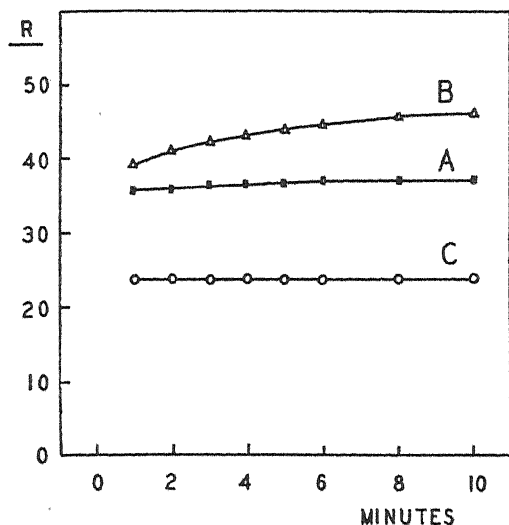


FIG. 3

Change during the Ten Minute Period Following the addition of Reagent to Cyanide and Alkali

- (A) Control.
- (B) Excess phenolphthalin.
- (C) Insufficient phenolphthalin.

quired for a complete reaction and presumably all of the substance is changed immediately to the phenolphthalein form. It is apparent that most of the troublesome spontaneous change in color which may follow the immediate cyanide reaction is eliminated by reducing the phenolphthalin concentration in the reagent to a minimum that still will give a complete reaction.

Influence of Oxygen. Some investigators have suggested that solu-

tions used in the test should be made up in boiled water so that spontaneous oxidation of the phenolphthalin by dissolved oxygen may be avoided (3). When there is not an excess of phenolphthalin in the reagent this precaution is apparently unnecessary. If a stream of oxygen is bubbled through a solution of 0.0001 *M* cyanide with optimum concentrations of alkali and reagent, the color curve for a ten minute period is almost identical with that of the control (Curve A). Even if the bubbling is continued for an hour period there is only a slight increase when compared to a control tube left standing in air.

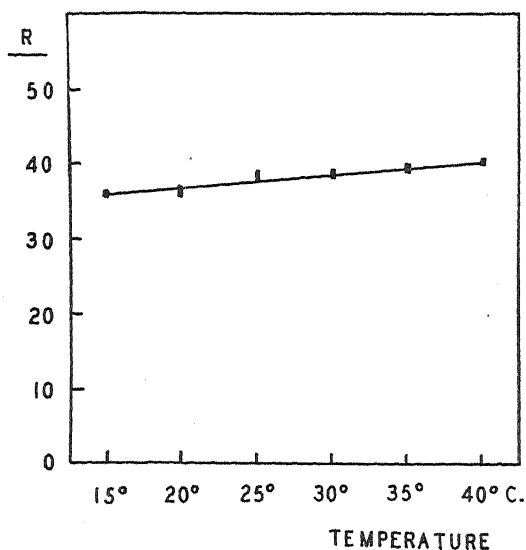


FIG. 4

Amount of Color Produced by Phenolphthalin Reaction with 0.0001 *M* KCN over a Temperature Range of 15°C. to 40°C.

Influence of Temperature. One of the phenolphthalin methods for cyanide determination (2), states that the test should be run at a temperature of 15° C. If there is excess of phenolphthalin in the reagent a slight change in temperature may cause considerable variation in the amounts of color which develop. With the improved reagent, however, this variation is reduced to a minimum. Fig. 4 shows that even though the temperature at which the reaction takes place is raised from 15° C. to 40° C., the amount of color produced increases only about 10%.

a standard concentration curve for cyanide is made at a temperature of 25° C. and at the time an unknown solution is determined the room temperature happens to be as low as 20° or as high as 30° C., the maximum error involved due to the temperature difference is only about 2.5%.

The Concentration Curve

Fig. 5 shows a curve obtained by plotting the electrophotometer readings of KCN solutions of 0.00001 *M* to 0.00015 *M* when treated with alkali and reagent as outlined. The curve is regular and nearly a straight line. For seven concentrations along this curve the maximum deviations within groups of ten identical samples were less than 3%.

The sensitivity of the determination is such that if the 2 ml. sample analyzed contains 0.5γ of CN' (1 part in 4,000,000), the maximum deviation expected is still within the 3% limit. One half of this quantity of cyanide, that is, one part in eight million, will still give a positive test.

Specificity

The phenolphthalin test is not entirely specific for cyanide: ferri-cyanides and aqueous solutions of halogens give a similar color (2), because of direct oxidation of the phenolphthalin. Interference may come from sulfides, which precipitate copper from the reagent, or from phenol (2), or ferrocyanides (6) that prevent full color development. Among those substances which are reported as not interfering with the reaction are halogen salts, H_2O_2 , ferric chloride, chromates, and HNO_3 (2). If there is uncertainty about the identity of the reacting compound the qualitative silver iodide test of Fox (7) may be employed. This is specific for cyanide and is nearly as sensitive as the phenolphthalin reaction.

METHOD

The standard procedure for the micro-determination of cyanide resulting from the above experiments is outlined below.

To prepare the reagent, 0.5 ml. of 1% phenolphthalin (Eastman Kodak Company) in absolute ethanol is added to 99.5 ml. of 0.01% $CuSO_4 \cdot 5H_2O$ and the two are mixed. This solution is stable for a day or more at room temperature and will keep in a refrigerator for months without change.

One ml. of 0.05% KOH is placed in a micro cell of a photoelectric colorimeter, 2 ml. of cyanide solution are added from a volumetric pi-

pette, and then 1 ml. of the reagent is added. The contents are mixed by inverting the tube several times and the color is determined immediately in the electrophotometer, using a #548 narrow range filter. The amount of cyanide in the unknown is then found by interpolating the reading on the logarithmic scale of the electrophotometer into a standard curve obtained with known concentrations of cyanide determined under identical conditions (see Fig. 5).

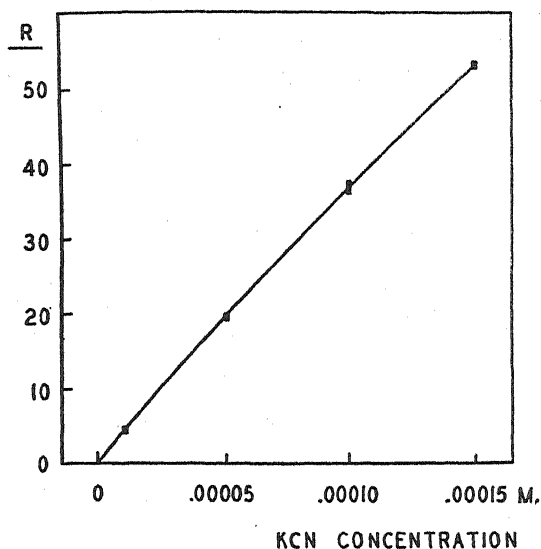


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5.86 per cent nitrogen. Turbidimetric and titrimetric bioassays of this salt gave an average value of 82.9 per cent compared with 85.8 per cent on the basis of the nitrogen analysis.

Adult male mice, C-57 strain, were used exclusively. This strain has been demonstrated to be comparatively resistant to pantothenic acid deficiency (7). The animals were depleted by placing them on a preliminary diet similar to that recommended by Unna and co-workers (8) for 8 to 12 weeks, during which they developed achromotrichia. After treatment with the labelled vitamin, the animals were exsanguinated by opening the carotid arteries. Each of the following organs or groups of organs were analyzed for total nitrogen and for N^{15} content: skin, heart, lungs, liver, kidney, gastro-intestinal tract, spleen and recovered blood, urogenital organs. The excess N^{15} was determined by the mass-spectrometric method of Rittenberg, *et al.* (9).

Values below 0.009 atoms per cent excess N^{15} were disregarded as being within the range of experimental error inherent in the determination of N^{15} and because of possible biological variations. Values from 0.009 to 0.010 were considered as traces; above 0.010 as available for quantitative evaluation.

TABLE I
Distribution and Excretion of p-Aminobenzoic Acid

Organ	Total Nitrogen mg.	Excess Atom N^{15} per cent	Total N^{15} γ
Skin.....	147	negative	—
Organs.....	177	0.009	traces
Carcass + tail.....	354	negative	—
Excreta and excess food.....	398	0.057	243

A. Retention and Excretion of p-Aminobenzoic Acid

A male mouse weighing 16 g., depleted as described, received a single intraperitoneal injection of 5 mg. of the labelled *p*-aminobenzoic acid. The mouse was sacrificed 24 hours after the injection and the N and N^{15} content of all organs was determined. The *p*-aminobenzoic acid if retained and uniformly distributed in the body of the mouse would have corresponded to a concentration of 0.010 per cent excess atom N^{15} . The determination of heavy nitrogen, however, revealed that the excess N^{15} values of all organs except the kidney were below 0.007 per cent. The kidney showed traces of excess atom N^{15} (0.009 per cent).

To study the influence of larger quantities of *p*-aminobenzoic acid, a male mouse weighing 22 g. received three subcutaneous doses of 10 mg. within a 24 hour period. This amount of labelled *p*-aminobenzoic acid if equally distributed in the organism would have resulted in an excess

atom N¹⁵ concentration equivalent to 0.045 per cent. Nineteen hours after the last injection, however, only traces were found in the organs but 227 γ N¹⁵ corresponding to 82 per cent of the injected amount were present in the excreta (see Table I).

B. Retention and Excretion of Pantothenic Acid

After preliminary depletion, a male mouse weighing 24 g. was injected subcutaneously, on each of 8 successive days, with labelled pantothenic acid totalling 155.5 mg. This was equivalent to 7.84 g. total nitrogen or to 784 γ N¹⁵. An equal distribution of this amount would have corresponded to a concentration of 0.108 per cent excess atom N¹⁵.

TABLE II

Distribution and Excretion of Pantothenic Acid After Subcutaneous Injection

Organ	Total Nitrogen mg.	Excess Atom N ¹⁵ per cent	Total N ¹⁵ γ
Liver.....	45.6	0.010	traces
G-I tract.....	67.6	negative	—
Blood plus heart plus spleen....	17.5	0.011	2.04
Kidney.....	10.6	0.012	1.39
Uro-genital tract.....	30.9	negative	—
Skin.....	158.3	negative	—
Carcass plus tail.....	386.6	negative	—
Lung.....	5.7	0.012	0.75

The experiment was terminated 48 hours after the last injection. The results (Table II) demonstrate that notwithstanding the large amount of pantothenic acid administered over a short time, only 0.5 per cent of the N¹⁵ injected was found in the organs.

DISCUSSION AND SUMMARY

No storage or utilization of labelled N¹⁵ of *p*-aminobenzoic acid has been demonstrable. These findings corroborate the results of Strauss and co-workers (10) obtained by colorimetric determination.

Rapid excretion by animals depleted of pantothenic acid follows its parenteral administration. Its almost complete absence in various organs indicates that the nitrogen of pantothenic acid, unlike that of amino acids, is not utilized by the organism. No storage of pantothenic acid in mice can be assumed.

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Effect of Glycerides of Hydroxy Fatty Acids upon Growth and Development¹

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INTRODUCTION

Saturated and unsaturated hydroxy fatty acids have been reported to be present in animal and vegetable fats, and thus are a component of foods, yet we have found no reports in the literature on the absorption, assimilation, deposition, or utilization of these acids by the animal body. Erber (1) has claimed that one-fifth of the fatty acid content of human chyle is hydroxy acids, and Artom (2) presented evidence that hydroxy acids constituted from 2% to 17% of the total fatty acids in lymph taken from the thoracic cavity of dogs during the absorption of a fatty diet. As the food fats did not contain these high percentages of hydroxy acids, it appears that the animal body can synthesize these fatty acids.

Hydroxy fatty acids have been isolated from the wax of conifers (monohydroxy lauric acid) (3), the fat of sheep wool (monohydroxy palmitic acid, lanoceric acid) (4), the wax of the cochineal (cocceric acid) (5), and from castor oil (dihydroxy stearic acid, ricinoleic acid) (6). The acetyl value of a fat or oil is generally accepted as a measure of hydroxy fatty acid content. Reports of the acetyl values of animal fats range between 7.5 in camel fat and 40 or more in wolf fat, running considerably higher in wild than in domestic animals (7). Butterfat contains significant amounts of these acids, especially dihydroxy stearic acid (8). It appears, therefore, that hydroxy fatty acids are constantly present in food fats and utilized as sources of energy. This paper reports the results of three series of experiments on three types of synthetic hydroxy

¹ This research was supported by a grant from Lever Brothers Company.

² Lever Biochemistry Fellow.

triglycerides (9), in an attempt to clarify the role played by hydroxy fatty acids in nutrition.

EXPERIMENTAL

Series I

Preparation of tri-dihydroxy stearyl glyceride. Dihydroxy stearic acid was prepared from oleic acid by the method of Lapworth and Mottram (10). The triglyceride was synthesized by reacting 600 g. of the dihydroxy stearic acid with the theoretical amount of c.p. glycerol in the presence of 0.2% NaOH catalyst, with strong agitation, under dry CO₂ to remove water, and in an oil bath at 200° C. for 6 hours. The triglyceride was washed repeatedly to remove soaps and free glycerol, then dried under vacuum (sap. val. 175.7, theory 170.6).

Preparation of tri-trihydroxy stearyl glyceride. Trihydroxy stearic acid was prepared from ricinoleic acid by the method of Lapworth and Mottram (10). This glyceride was synthesized by reacting 580 g. of trihydroxy stearic acid with the theoretical amount of c.p. glycerol at 210° C. oil bath temperature with rapid agitation and no catalyst. After 6 hours the triglyceride was washed and dried under vacuum (sap. val. 171.4, theory 162.7).

Month-old Wistar-strain albino rats were housed separately in galvanized wire cages and supplied with water and diets, *ad libitum*.

Two groups of 12 male rats each were fed diets I and II for a period of 175 days. Later, two additional groups of 14 male rats each were fed diets III and IV for a period of 87 days. The composition of these diets is given in Table I. Records were kept of the weight increase and food consumption of each rat. At the end of each experimental period the animals were decapitated and the tissues and organs examined for gross evidence of pathological change. None were observed.

Beginning with the 21st day the group fed the tri-dihydroxy stearyl glyceride increased in weight more rapidly than that fed the control hydrogenated fat (Curves I and II, Fig. 1), so that by the end of 96 days this test group averaged 25.8 g. (or 9%) heavier. This was not due to greater food intake because this group consumed 8% less food (4.4 vs. 4.8 g.) per gram gain in body weight than the control group.

From the beginning, the group fed the tri-trihydroxystearyl glyceride showed less gain in body weight than the control group. At the end of the test period the average difference was 5.7 g. or 2%. However, since the test group consumed approximately 5% less food (4.4 vs. 4.6 g.) per gram gain in body weight, the performance of this group was actually slightly superior to that of the control.

These experiments with two types of hydroxy fatty acid triglycerides

show them to be non-toxic when fed as glycerides and mixed with dietary fat. The tri-dihydroxy stearyl glyceride produced an improvement in the growth of rats when fed at a level of 2.2% in replacement of an equivalent weight of hydrogenated fat in a complete dietary. Tri-trihydroxystearyl glyceride is not as effective in this respect. These superior nutritional results were obtained with triglycerides having high melting points (70° C. and 90° C.).

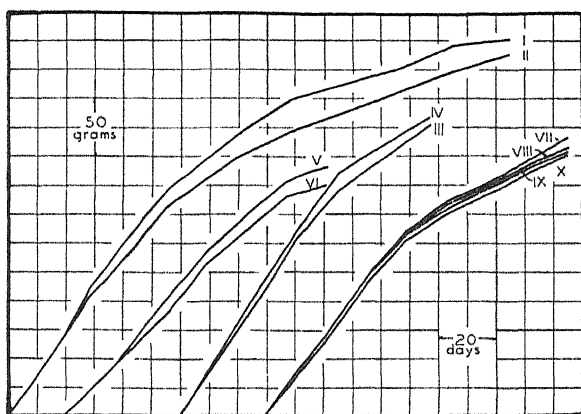


FIG. 1

Curves Presenting Weight Increases of Rats Fed Hydroxy Fatty Acid Triglycerides

Groups were fed diets containing the following amounts and kinds of triglycerides: I, 2.2% tri-dihydroxy stearyl glyceride; III, 2.2% tri-trihydroxy stearyl glyceride; V, 2.5% tri-dihydroxy stearyl glyceride; VII, VIII and IX, 2, 1, and 0.5% mono-dihydroxy stearyl triglyceride respectively. II, IV, VI and X were controls.

Analyses of the body tissues of Groups I and II showed no difference in the water or fat content, indicating that the greater weight of the animals on the tri-dihydroxy stearyl glyceride was not due to increased deposition of adipose but rather to better growth.

Series II

Because of the favorable results with tri-dihydroxy stearyl glyceride, this investigation was repeated on two groups of 13 rats each, with some modifications in the diet (diets V and VI, Table I). The rats were housed and fed as in the previous experiments and weighed weekly over

a period of 91 days. The weight increases of these groups are shown in Fig. 1 (Curves V and VI).

After the animals were killed by decapitation, the hide of each animal was removed and combined with those of all animals in the same group. The adipose tissue, freed from the internal organs, was added to the carcass (muscle and skeleton) sample. The internal organs were discarded.

Each combined carcass sample was passed through a meat grinder, then placed in 2000 ml. of acetone,³ refluxed for 8 hours, then allowed to

TABLE I

The Composition of Diets Employed in This Investigation

Diet:	I	II	III	IV	V	VI	VII	VIII	IX	X
Number of rats.....	12	12	14	14	13	25	20	20	20	20
Extracted casein (Labco)....	25%	25%	25%	25%	18%	18%	18%	18%	18%	18%
Sucrose.....	40	40	40	40	53	53	53	53	53	53
Salt mixture (Hubbell).....	5	5	5	5	4	4	4	4	4	4
Brewer's yeast (Harris).....	8	8	8	8	—	—	—	—	—	—
Hydrogenated fat (Spry)....	19.8	22	19.8	22	22.5	25	23	24	24.5	25
Tri-dihydroxystearyl glyceride.....	2.2	—	—	—	2.5	—	—	—	—	—
Tri-trihydroxystearyl glyceride.....	—	—	2.2	—	—	—	—	—	—	—
Tri-glyceride containing 33% of fatty acids as dihydroxystearic acid.....	—	—	—	—	—	—	2	1	0.5	—
Daily vitamin supplement...	a, b	a, b	a, b	a, b	a, c	a, c	a, c	a, c	a, c	a, c

Vitamin supplement a = 60 I.U. vitamin A, 6 I.U. vitamin D in mineral oil (Nujol).

Vitamin supplement b = 5 mg. choline hydrochloride in aqueous solution.

Vitamin Supplement c = 20 γ thiamin, 25 γ riboflavin, 100 γ dicalcium pantothenate, 20 γ pyridoxin, 100 γ niacin, 15 mg. choline in aqueous solution.

stand overnight before removing the solvent. This acetone extraction was repeated twice more, then twice with U.S.P. ethyl ether.³ The solvents were removed from the lipid extracts by distillation under nitrogen and the residue dried to constant weight at 45° C. in a vacuum oven. The alcohol and ether extracts were combined, then diluted to 2000 ml. with acetone.³ The acetone-insoluble fraction (consisting mainly of phospholipids) was removed by centrifugation so that the acetone-soluble lipid content of an aliquot of the supernatant acetone could be determined (see Table II).

³ Redistilled from KOH.

The hide samples were submitted to the same extraction procedures except that 1000 ml. quantities of acetone or ethyl ether were used.

A quantitative analysis was made of the carcass fat obtained from both groups, using the procedure of Hilditch (9) in which 100 parts by weight of fat are saponified for 4 hours with a solution containing 120 g. of KOH per liter of 95% ethyl alcohol. After saponification most of the alcohol was removed, the soaps taken up by water, then extracted with ether for 24 hours using a continuous extractor. After the unsaponifiable matter had been removed by this treatment, the soaps were acidified with dilute H_2SO_4 and the steam-volatile acids removed by steam

TABLE II

The Composition of Dietary Fats Used in Part of This Research and the Composition of Tissue Fats of Rats Fed Diets Containing Them

Diet.....	Carcass		Hide		Diet fat	
	V	VI	V	VI	V	VI
Number of rats.....	13	25	13	25		
Wet weight (g.).....	1536	1822	377	467		
Neutral fat (g.).....	174	183	45.5	81		
% of tissue.....	12	10	13	17		
Sapon. value.....	182.1	183.0	182.9	175.0	194.5	195.2
Iodine value.....	72.9	77.9	71.8	73.0	57.8	65.7
Thiocyan. value.....	66.8	67.8	65.5	66.4	55.4	56.2
Acetyl value.....	15.6	9.8	—	—	36.2	4.1
Unsaponif. (%).....	0.8	0.9				
Steam vol. acids (%)*	0.2	0.1				
Mixed fatty acids (%)..	89.8	90.0				
Saturated acids.....	29.3	37.5				
Unsaturated acids.....	60.5	52.5				

* Calculated as butyric acid.

distillation. The residual fatty acids were extracted with ether, then recovered by removing the ether by distillation and drying in a vacuum oven. The mixed fatty acids were then converted into their lead salts and crystallized from 95% ethyl alcohol at 15° C. The fatty acids were regenerated from their lead salts by treatment with HCl. The results are reported in Table II.

The group fed the dihydroxy glyceride was 15 g. (or 6%) heavier than the control group at the end of the 91-day period. Since this group consumed 26% less food (7.0 vs. 5.2 g.) per gram body weight increase, it again appears that the addition of 2.5% tri-dihydroxy stearyl glyceride improved the growth-promoting properties of a nutritionally complete

dietary. There was a definite tendency for the dietary fat to influence the character of the fat of the carcass and hide. The group fed the hydroxy-fat diet deposited definitely less fat in the hide and slightly more fat in the carcass.

Series III

The tri-dihydroxy stearyl glyceride used in Series I and II exerted its beneficial effect in spite of a melting point (70°C.) so high that one might expect it to be indigestible. Better results might be expected with a glyceride of much lower melting point, one in which each molecule contained one, rather than three, hydroxy acids.

Preparation of triglyceride containing 33% of fatty acids as dihydroxy stearic acid. Sodium dihydroxy stearate was added to a batch of hydrogenated fat (Spry) which had previously been saponified with NaOH, in amounts equivalent to one-third the calculated fatty acid content of the saponified mixture. The batch was then esterified at 200°C. The resulting fat thus contained an average of one molecule of dihydroxy stearic acid in each glyceride molecule: m.p. less than 37°C. , Sap. No. 189.

One gram of tri-dihydroxy stearyl glyceride is equivalent to approximately three grams of mono-dihydroxy stearyl triglyceride in hydroxy acid content. Thus a diet containing 6.6% or 7.5% of the latter triglyceride is as rich in hydroxy acid content as diets I and V. It was realized, however, that the tri-dihydroxy stearyl glyceride could not have been completely digested and absorbed because of its high melting point (70°C.). Thus it was decided to feed the mono-dihydroxy stearyl glyceride at definitely lower amounts, using several levels in an attempt to quantitate its effectiveness.

Four groups of 20 male rats each were used in this series. One group received control diet X (Table I), the same as that fed to Group VI. The remaining three groups were fed diets in which 2.0, 1.0, and 0.5% mono-dihydroxy stearyl triglyceride had replaced equivalent weights of hydrogenated fat (diets VII, VIII, and IX, Table I). The acetyl values of the dietary fats in diets VII to X were 12.5, 7.6, 6.1, and 4.1 respectively.

This experiment was continued for 105 days by the same technique and the same conditions as in Series II.

The weight increases of these four groups (VII to X) are shown in Fig. 1. The group fed the 2% level of hydroxy glyceride gained an

average of 16 g. (7%) more, the group fed the 1% level of hydroxy glyceride averaged 7 g. (3%) more, and that fed the 0.5% level averaged 3 g. (1%) more, than the control group. The diet consumption averaged 7.1, 7.2, 7.4, and 7.5 g. of diet per gram of body weight increase in Groups VI to X respectively.

SUMMARY

1. A synthetic glyceride containing all of the acids as dihydroxy stearic acid was fed in nutritionally complete diets to weanling rats at levels of 2.2% and 2.5% in replacement of equal weights of hydrogenated fat in control diets. The rats fed the hydroxy glyceride gained more weight during test periods of 105 days and 175 days, and showed superior gain in weight per gram of food intake, due to superior growth and development and not excess deposition of adipose, for the fat content of both groups was the same. There was a tendency for the rats fed the hydroxy glyceride to deposit less fat in the dermal tissues and for the control rats to deposit less fat in the carcass (muscle tissues, bones, and peritoneum). The hydroxy fat influenced the composition of the carcass fat of the rats analyzed after 105 days but had no measurable effect after 175 days.

2. A similar experiment with tri-trihydroxy stearyl glyceride fed in a diet at a level of 2.2% indicated that this hydroxy glyceride had little if any capacity to influence the growth and development. The group fed this glyceride showed a better gain per gram of food intake, but the total grams gained was less, than that of the control group. The difference in effectiveness of tri-trihydroxy and tri-dihydroxy stearyl glycerides may be due entirely to differences in melting point (90°C. vs. 70°C.).

3. Mono-dihydroxy stearyl triglyceride was fed at levels of 0.5%, 1.0%, 2.0% in nutritionally complete diets of rats in the same manner. All levels of this hydroxy glyceride favorably affected the growth and development, in direct proportion to the amounts fed. In each instance, greater growth was achieved on a lower intake of food than the control group. As this glyceride contained approximately one-third as much hydroxy acid as the tri-dihydroxy stearyl glyceride, it is evident that the mono-dihydroxy triglyceride is the more active, presumably due to its greater solubility and digestibility.

4. Hydroxy fatty acid glycerides exert a favorable effect upon the growth and development of rats on dietaries already presumed to be adequate.

Acknowledgments

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Hydrogen Ion Concentration of Thick and Thin White of Eggs*

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During a study of the nature of "watery whites," determination of the pH of the thick and the thin portion of egg white from both fresh and storage hens' eggs has been made. Sharp and Powell (1931) obtained a value of 7.6 for the pH of fresh egg white. Several other workers, however, have obtained somewhat higher values. Smith (1934), working in England, reported a pH value of 7.97 for the white of fresh eggs. Healy and Peter (1925), using a colorimetric method, obtained a value of 8.24. Ulrik and Davidsen (1933) reported 8 to 8.2 as the normal pH value of the white of eggs at laying. Erikson, Boyden, Martin, and Insko (1932) found 7.92 for white from fresh eggs. St. John (1936) reported preliminary observations showing the pH value to be around 8.0, and a lack of equilibrium between the thick and the thin portions of the white. None of these authors expect Erikson, Boyden, Martin, and Insko (1932) and St. John (1936) reports a separation of the thick and thin portions of the white.

In view of the results by different investigators additional measurements of pH on the white of fresh eggs have been made in these laboratories over a period of more than 10 years. Each egg was broken onto a watch glass, and the thick and thin portions of the white were separated by pouring from a beaker to a watch glass and back several times as described in more detail by St. John and Green (1930). This method is preferred to the use of a screen, because of less tendency to disrupt the colloidal structure of the thick white. Measurements were made on individual eggs soon after breaking, without first mixing the white, by

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inserting the electrodes directly into the white immediately after separation from the yolk. Measurements were made on fresh eggs, that is those not more than one hour old, on eggs less than four hours old, on eggs held at room temperature from one to three days, on those held in the ice-box from one to five weeks, and on eggs held in cold storage for two and for three years. In many cases, measurements were made on eggs immediately after laying which were still warm. In 1943, all meas-

TABLE I
pH of Albumen in Fresh Eggs

	Bailey electrode		Beckman pH meter					
	1931		1938		1939		1943	
	Thin	Thick	Thin	Thick	Thin	Thick	Thin	Thick
Mean.....	8.05	7.95	8.11	7.91	7.84	7.73	8.15	8.07
Extremes.....	7.66	7.52	7.75	7.56	7.50	7.44	7.87	7.59
	8.35	8.15	8.61	8.23	8.36	8.11	8.58	8.38
PEs ¹	0.10	0.11	0.10	0.12	0.11	0.09	0.11	0.12
PEm ²	0.024	0.030	0.012	0.013	0.009	0.008	0.020	0.025
n.....	18	18	79	79	142	142	32	32

Age Four Hours

Mean.....	7.89	7.73
Extremes.....	7.71	7.52
	8.08	7.96
PEs ¹	0.068	0.097
PEm ²	0.014	0.020
n.....	23.0	23.0

¹ PEs, probable error of a single determination.

² PEm, probable error of the mean.

Appreciation is gladly expressed to Russell Edwards, R. Parrish, and Andrew Leiser for laboratory assistance.

urements were made on eggs which were still warm and in some cases by breaking a small hole in the shell and inserting the electrodes into the white directly through this hole. The results on broken out fresh eggs using the Bailey electrode, and the Beckman pH meter are shown in Table I.

The data in Table I show a pH value for the white of fresh eggs centering around 8.0. This value is supported by colorimetric measurements

made in this laboratory. A few measurements on individual eggs reached a value as low as 7.6 which was believed by some earlier workers to be the pH of fresh egg albumen. The averages on eggs which had stood for four hours varied little from those on fresh eggs.

From Table I it appears that the thick and thin portions are not in equilibrium. The average difference between the two portions is above 0.13 pH. The average value obtained for the thin white is above that for the thick white both on fresh eggs and those which have stood for a few hours, although the variation in individual eggs is not always in this direction. Some results showed a difference between thick and thin which was in the opposite direction. In the case of thirty-two out of 142 eggs included in the 1939 measurements the pH of the thick portion was higher than that of the thin portion. Averaging the results for this latter group we find that the pH of the thin was 7.72 while that of the thick was 7.82, an average difference of approximately 0.1 pH. Eliminating these results from the entire group leaving the measurements on 109 eggs which showed a higher value for the thin than for the thick, we find the average results to be 7.88 for the thin and 7.70 for the thick portion, a difference of approximately 0.18 pH. As noted above, the average overall difference for the entire group was above 0.13 pH. The maximum range between eggs is comparatively large. It seems evident from the data presented that the thick and the thin portions of the white are not in equilibrium. This might be expected in view of the colloidal nature of the white, particularly the thick viscous condition of the thick white which may be described as a gel structure. The attainment of equilibrium would naturally be retarded.

Variability between individual eggs is supported by data of other types such as those presented by St. John and Flor (1930), St. John (1936), and St. John and Caster (1944). Whether or not such variability is definitely related to other factors is not evident. No definite relation to relative whipping ability or to the percentage of bound water in the thick and thin portion of the white appears, although the thin white which is on the average more alkaline, appears to have a smaller percentage of bound water and to give a larger volume upon whipping. It is probable that the variation in this initial pH value may be of comparatively little importance in relation to the keeping quality of eggs since the pH in all eggs rapidly increases to higher values in a few days. As eggs age the whites increase in alkalinity and show less differences between thick and thin white. After three days at room temperature

the pH of the thin was 9.33 and of the thick 9.40. If the eggs are stored in the ice box there is little further increase in pH, and at the end of five weeks the pH of thin was 9.34 and thick 9.35.

Eggs were held in cold storage for two years at a temperature maintained close to freezing. The eggs which were in the best storage condition had the most alkaline reaction, the pH of the thin being 9.00 and the thick 8.72, while those that were not in such good condition were less alkaline, the thin having a pH of 8.33 while the thick was 8.10, but in all cases the pH of the thick was less than that of the thin.

The eggs which had been in storage for three years had deteriorated to such an extent that sufficient white to make measurements was obtained from only three eggs. No separation of the thick and thin portions could be made. The average pH for the three determinations was 8.10. One egg which appeared much the same as the two year old eggs had a pH of 8.23; while another egg from which only a small amount of jellied white could be obtained had a pH of 7.76. This suggests that deterioration in the egg is accompanied by increasing acidity.

It has been rather commonly believed that the increase in the pH of egg white with time after laying is due to the loss of carbon dioxide from the white. Sharp and Powell (1931) and others have advocated this theory and Sharp refers to Scholl (1893), who claimed to have separated carbon dioxide from egg white. Erikson, Boyden, Martin, and Insko (1932) appear to accept this explanation. Swenson (1938) accepted this explanation on the basis of measurements by titration. Sharp and Stewart (1931) emphasized the apparent effect of storage in an atmosphere of carbon dioxide. Perhaps the most critical and scientific consideration of this hypothesis has been developed by Brooks and Pace (1938), who assume that the change in the pH of the egg white is due to carbon dioxide originally contained in the white, based on the relation of the pH changes calculated and the partial pressure of (HCO_3). They point out, however, certain contradictions based on presumed carbon dioxide content of the thick and thin portion of the white in relation to pH changes. The Erikson, Boyden, Martin, and Insko statement regarding a rapid loss of carbon dioxide from the white is difficult to understand, since they found little change in the pH of the white of eggs which had stood up to nine hours, and since the pH did not change in the white which had stood for thirty minutes after the egg white had been separated out. In fact the pH of their eggs decreased by 0.2 in one hour after laying and did not return to the original value of 8.0 until the eggs had stood for more than eight hours.

In the data presented in Table I above, the results for 1939 show that the pH of the white after the eggs had stood for four hours was nearly identical with that of eggs immediately after laying. More recent measurements are presented in Table II, where the pH was determined

in eggs which were still warm by breaking a small hole in the shell and inserting small glass electrodes. The egg was later broken, the white separated, and the measurements of pH were again made. Each group in Table II represents six or more fresh eggs immediately after laying. The average in each group is consistently as high or higher in the shell than in the separated white, and the average of the four groups indicates that the pH value of the white still in the shell was nearly 0.1 higher than after the white was separated out.

The results in Tables I and II, and the data by Erikson, Boyden, Martin, and Insko (1932), contradict the possibility of a direct relationship between pH and the carbon dioxide content of the white, in addition to the contradiction noted by Brooks and Pace (1938). Further, the conclusion of Brooks and Pace that the pH of the white is influenced by the presence of carbon dioxide in the white is based upon indirect

TABLE II
pH of White in Shell and After Separation

Group	In Shell	White	Thin	Thick	Yolk
1	8.20	8.09	8.06	8.01	6.11
2	8.13	8.13	8.12	8.06	6.11
3	8.29	8.11	8.23	8.19	6.14
4	8.31	8.28	8.29	8.12	6.13
Ave.	8.23	8.15	8.15	8.07	6.13

evidence, or is an assumed relation between the change in the partial pressure of carbon dioxide and a change in pH.

Consider the relation of the following facts to the possibility that a change in carbon dioxide content might be a factor of importance in causing a change in pH. Carbon dioxide is only slightly soluble. It is a weak acid, that is only slightly ionized. Egg white is a well buffered system. The white is rather highly viscous, tending to retard any rapid escape of carbon dioxide. The white is surrounded by a semi-permeable membrane and by a shell, both of which would retard any rapid escape of carbon dioxide, even if pressure or temperature conditions were favorable to such escape. These considerations would seem distinctly unfavorable to the rapid loss of any material quantity of carbon dioxide from the white. Further, even if a material quantity of carbon dioxide should be lost from the white, it would seem incapable of causing the comparatively enormous change in pH which does occur. On first thought, loss of carbon dioxide from the white may seem an obvious and

quite logical explanation of changes in pH. However, the evidence in support of the hypothesis is largely, if not entirely, indirect evidence. In contrast to that, there is evidence as described above, indicating that loss of carbon dioxide is not the cause of rapid and large changes in pH after the egg is laid. It appears that more direct and positive proof is essential before the hypothesis can be accepted.

SUMMARY

Hydrogen ion determinations were made on the thick and thin portions of egg white of fresh eggs and on those stored under different conditions, and for different lengths of time. The hydrogen ion concentration of eggs of different consistency, varying from firm to watery, is practically the same. In the fresh egg the initial pH varied over a fairly wide range, centering around an average of 8.0. This is higher than the results reported by early workers but it is confirmed by more recent work using improved methods of measurement. These results, as well as data of other types, emphasize the variability between individual eggs. In a large proportion of instances a difference between the thick and thin portions appeared. Equilibrium between the two portions is not established. After storage for five weeks there was little or no difference between thick and thin. After two years storage there was again a difference in pH between the thick and thin portions. The thick was less alkaline than the thin portion, the pH reading of the thin being 9.00, and the thick 8.72. After three years storage the pH value was 8.01, and no separation of thick and thin portions could be made. Evidence is presented indicating that a loss of carbon dioxide with time of standing may not be the explanation of the increase in pH which occurs.

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The *cis-trans* Isomerization of α -Carotene Isomers¹

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INTRODUCTION

During the past two years Zechmeister and coworkers have published results of extensive studies on the isomerization of carotenoids (1). Their work has elucidated many problems in this field and has also raised many new questions regarding the chemistry of these interesting isomeric groups. At the time this work was undertaken *cis-trans* isomerization of lycopene, γ -carotene and β -carotene had been reported in some detail. The case of α -carotene presents a slightly different system of conjugation, and a study of this stereoisomeric set was undertaken. Since the completion of the experimental part of this work, a detailed report on the α -carotene stereoisomeric set has been published by Zechmeister and Polgár (2). The present paper contains more detailed absorption data in the visible region for certain isomers of α -carotene than were reported by Zechmeister and Polgár (2) and extends their work on heat isomerization. In addition, much of their experimental work is herein confirmed.

EXPERIMENTAL

Heat Isomerization

The technique used in the heat isomerization experiments differs essentially from that used by Zechmeister (2) in that the solutions were refluxed in hexane (65°) for 18 hours instead of only 30 minutes. The longer time was chosen in the hope that a heat isomerization equilibrium mixture would be obtained, but continued changes after even longer periods of time made it apparent that an equilibrium was not reached.

In a typical heat isomerization, 19 mg. of α -carotene was dissolved in 150 ml.

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of warm hexane and the solution boiled in darkness under a reflux condenser for 18 hours.

In a second series of heat isomerization experiments, isooctane (2,2,4-trimethyl-pentane—b.p. 99°) was used as the solvent to permit the convenient use of a higher temperature.

Iodine-Light Isomerization

In a typical iodine-light isomerization experiment, 4.4 mg. of α -carotene was dissolved in 200 ml. of hexane containing 2.5% ethyl ether (to increase solubility) and 0.08 mg. of iodine dissolved in hexane was added. The solution was exposed to an illumination of 30 f.c. (measured with Weston Illumination Meter, Model 603) from a 60 watt incandescent lamp at 25° for 30 minutes. To permit better comparison with heat isomerization mixtures, the isomerization was allowed to continue until the same reduction in light absorption at 4720 Å was effected as was accomplished by 18 hours boiling in hexane solution. 4720 Å was chosen because of its proximity to the longer wave length maxima of both all-*trans* α -carotene and the resulting isomerization mixture.

At the end of the exposure time, the hexane solution was extracted four times with 20 ml. aliquots of a tenth molar solution of $\text{Na}_2\text{S}_2\text{O}_3$ to remove the iodine and preclude the possibility of further catalytic change. Before chromatography the solution was washed four times with water to remove the thiosulfate.

General

Crystalline chromatographically and spectroscopically pure α -carotene was employed.

Alumina was used as an adsorbent in all these studies and the columns were developed by use of 2% ethyl ether in hexane. After development of a chromatogram the zones were separated and the pigment eluted with 5% ethanol in hexane. After elution the pigment solutions were washed with water, dried over anhydrous Na_2SO_4 , made to volume, and examined spectroscopically. The pigment from each zone was adsorbed on a series of alumina columns until no further purification could be effected by this chromatographic technique. Reproducibility of spectral absorption curves and homogeneity on alumina columns were used as criteria of purity. The system of nomenclature used by Zechmeister and Polgár (2) is employed here to designate pigments of different zones.

In all chromatographic work the columns were cooled by a jacket through which dry-ice-cooled acetone was circulated. The temperature inside the columns was thus kept at about -10° . This precaution was taken to minimize the possibility of changes taking place while the pigments were on the column.

Spectral absorption measurements were made with the same photoelectric spectrophotometer employed under the same conditions as used for previous studies on carotenoid spectra, and solvents were purified according to methods previously described (3). Absorption coefficients were calculated by use of density values ($\log \frac{I_0}{I}$ calculated to the base 10).

Attempts at crystallization of two of the isomers proving unsuccessful, this method was abandoned and the ordinate values of the characteristic curves were

determined by heat isomerization of isomer solutions in a manner similar to that employed by Beadle and Zscheile (4). Since in many cases the all-*trans* isomer was the principal one produced, the point on the characteristic curve which was not changed by this treatment was taken as a coincident point of the characteristic curve of the isomer with that of all-*trans* α -carotene. This method is subject to error to the extent that heat isomerization results in total destruction in so far as absorption is concerned, and to the extent that reverse isomerization yields pigments having absorption values different from that of the all-*trans* isomer at the point selected as the coincident point. (In this paper the term "reverse isomerization" indicates isomerization of any *cis*-isomer, the all-*trans* isomer being considered as the parent substance.) Since only small amounts of the all-*trans* isomer were produced by heat isomerization of the mixture of neo α -carotenes U, V, W,² the coincident point for this mixture was determined from iodine isomerization curves. The errors of this method are somewhat minimized because the curves of the principal isomers neo U, V, W, neo C, and neo B all have apparent coincident points at 4000 Å.

RESULTS

Heat Isomerization

The following chromatogram is typical of an isomerization mixture obtained after 18 hours refluxing in hexane solution (on the left the zone width is given in millimeters; the widths of the bands are not indicative of the relative pigment concentrations in the original solution):

Chromatogram of Mixture from Heat Isomerization at 65°

20—light yellow	} probably oxidized pigments
10—light red	
140—orange—unchanged all- <i>trans</i> + trace neo U, V, W	
120—light orange—neo C	
40—colorless interzone	
120—yellow—neo E	

Heat isomerization at 99° yielded a different group of isomers than the principal isomers obtained at 65°. A typical chromatogram from such a mixture is shown below:

Chromatogram of Mixture from Heat Isomerization at 99°

10—light yellow	} oxidized pigments
20—light red	
100—orange	{ 50—neo U, V, W 50—unchanged all- <i>trans</i>

² These three isomers were not adequately separated by the technique employed here and are designated as a group.

³ Separations obtained on a second chromatogram of the isolated original zone.

80—light orange $\begin{cases} {}^3\text{neo B} \\ \text{neo C}' \end{cases}$
 50—yellow—neo D

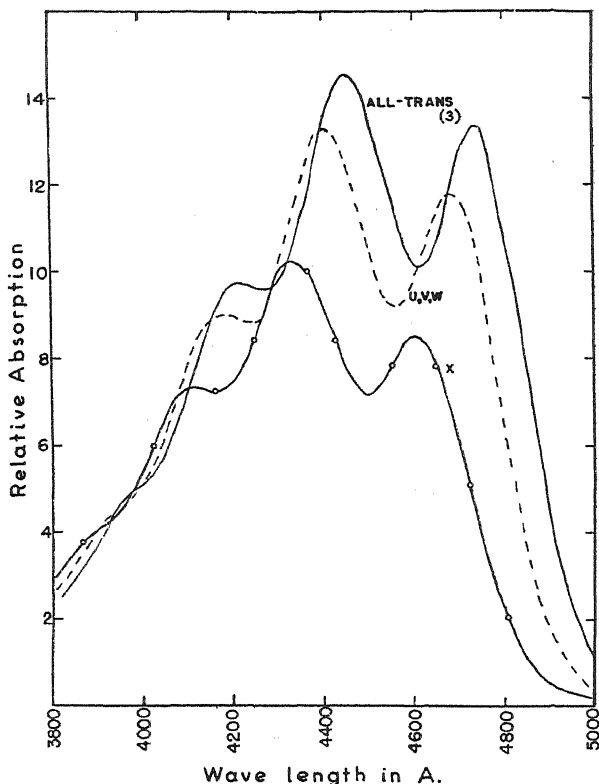


FIG 1
 Absorption Spectra of α -Carotene Isomers in Hexane

Iodine-Light Isomerization

Almost exactly the same chromatographic picture was obtained from iodine-light isomerization mixtures as from 99° heat isomerization mixtures. This is illustrated by comparison of the 99° chromatogram with the chromatogram illustrated below from a typical iodine-light isomerization mixture.

³ See footnote p. 79.

Chromatogram of Mixture from Iodine-Light Isomerization

10—light yellow } oxidized pigments
 10—light red }
 80—orange { ³40—neo U, V, W
 40—unchanged all-*trans*

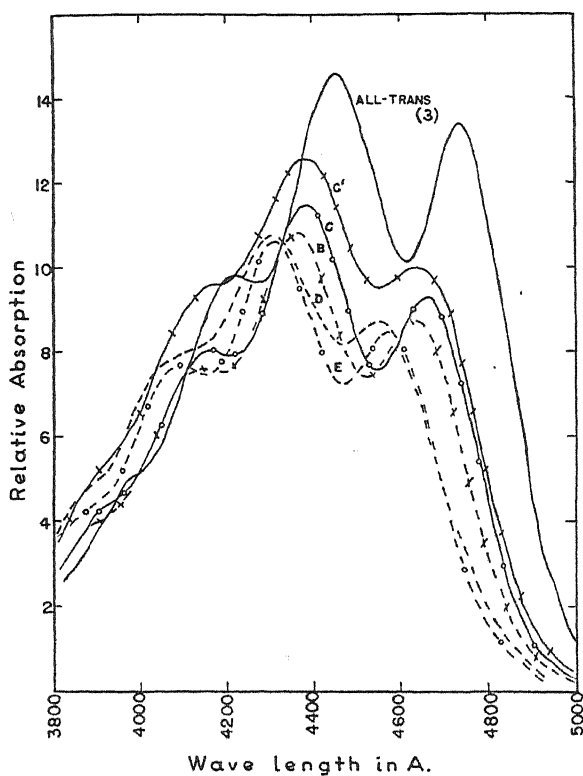


FIG. 2

Absorption Spectra of α -Carotene Isomers in Hexane

150—light orange—neo B

100—buff—neo C'

50—yellow—neo D

The absorption curves of the isomers studied are shown in Figs. 1 and 2.

³ See footnote p. 79.

Absorption curves of some of the isomers in the ultraviolet region are shown in Fig. 3. The differences in relative absorption at the *cis* peak between the all-*trans* form and neo U, V, W, neo B, neo C, and neo E

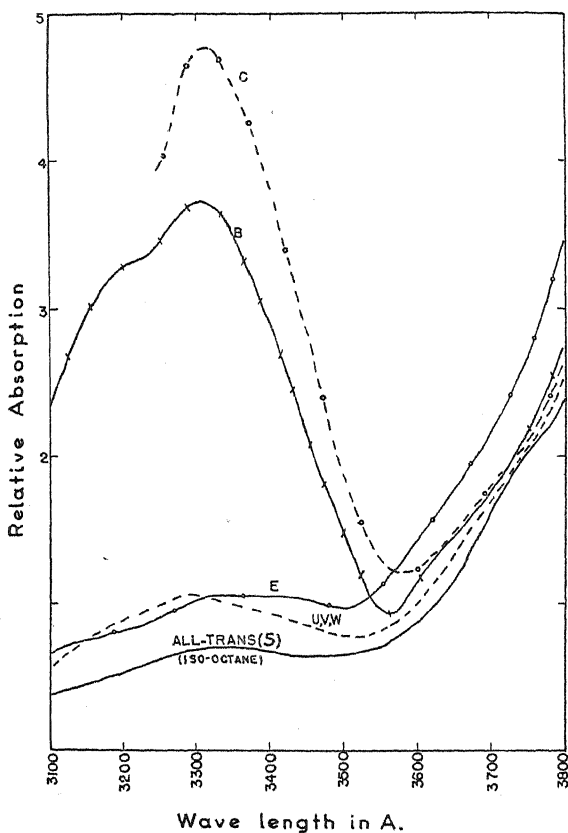


FIG. 3

Ultraviolet Absorption Spectra of Certain Isomers of α -Carotene in Hexane

Accurate data for all-*trans* α -carotene in hexane were not available in the ultraviolet region. Considerable data indicate that almost identical values would be obtained for iso-octane and hexane as solvent.

are 0.3, 3.0, 4.1, and 0.3, respectively. The curves were placed by the coincident point technique described above. The uncertainty of this method has prompted us to designate the ordinate values in terms of

relative absorption. These values ($\times 10^4$) are numerically equal to molecular absorption coefficients within the limits of accuracy of the method used in their determination. Relative absorption values at maxima, minima, and important coincident points with the all-*trans* isomer are presented in Table I. It will be noted that in many cases

TABLE I
Spectroscopic Data on α -Carotene Isomers

Isomer	Maxima		Minimum	Coincident points with all- <i>trans</i> α -carotene used in establishing curve positions	
Neo U, V, W	4680 Å	4400 Å	4560 Å	4000 Å	4390 Å
	<i>11.8*</i>	<i>13.3</i>	<i>9.22</i>	<i>5.15</i>	<i>13.2</i>
Neo X	4600	4330	4500		4100
	<i>8.54</i>	<i>10.3</i>	<i>7.21</i>		<i>7.29</i>
All- <i>trans</i> (3)	4740	4460	4615		
	<i>13.3</i>	<i>14.6</i>	<i>10.1</i>		
Neo B	4640	4360	4520		4000
	<i>8.74</i>	<i>10.8</i>	<i>7.40</i>		<i>5.15</i>
Neo C	4660	4380	4550		4000
	<i>9.34</i>	<i>11.5</i>	<i>7.56</i>		<i>5.15</i>
Neo C'	4640	4380	4560		4380
	<i>10.0</i>	<i>12.6</i>	<i>9.5</i>		<i>12.6</i>
Neo D	4550	4300	4470	4120	4330
	<i>8.75</i>	<i>10.8</i>	<i>8.15</i>	<i>8.04</i>	<i>10.5</i>
Neo E	4580	4310	4470	4110	4330
	<i>8.47</i>	<i>10.6</i>	<i>7.18</i>	<i>7.61</i>	<i>10.5</i>

* Italicized figures are molecular absorption coefficients ($\times 10^{-4}$) in liters per mol centimeter, with accuracy for all isomers except the all-*trans* limited by errors in determination of the coincident points.

the maxima and minima occur at shorter wave lengths than those reported by Zechmeister and Polgár (2). However, corresponding wave length shifts from the all-*trans* parent isomer at the longer wave length maxima agree well. The maxima and minima in the visible region reported by Zechmeister and Polgár [Table I (2)] were determined visually

and do not agree with their similar data obtained with the Beckman spectrophotometer for neo U (2) and for the all-*trans* isomer (6). Such disagreements indicate that the visually determined wave lengths for the

TABLE II
Yield of α -Carotene Isomers by Heat and Iodine-Light Isomerizations
Percentage of starting material appearing as

Starting material	Treat-ment	Neo U, V, W	Neo X	All- <i>trans</i>	Neo B	Neo C	Neo C'	Neo D	Neo E	Neo unidentified*	Total
Heat	18 hrs. at										
Neo, U, V, W.....	65°	57	13	2.3	— ^Δ	—	—	—	—	5.0	77
Neo X.....	65°	53	—	2.5	—	—	—	—	—	—	55
All- <i>trans</i>	65°	3.5**	—	56	—	20	—	—	1.5	—	81
All- <i>trans</i>	99°	26	—	43	18	—	6.6	2.2	—	—	95
Neo B.....	65°	20	—	30	15	—	—	—	—	6.5	71
Neo C.....	65°	2.6	—	64	—	9.0	—	—	—	14	89
Neo C'.....	65°	16	—	64	—	—	16	—	—	—	96
Neo D.....	65°	19	—	33	—	—	—	—	—	—	52
Neo E.....	65°	3.2	—	29	—	10	—	—	—	—	42

Iodine-light

Neo U, V, W.....	2 hrs. exposure	12.0***	—	7.5***	7.0***	—	—	—	—	2.0	28
All- <i>trans</i>	absorption reduced 13% at 4720 A.	29	—	45	9.4	—	1.7	—	—	0.7	95
All- <i>trans</i>	Absorption reduced 24% at 4720 A	27	—	33	20	—	1.5	0.9	—	—	82

* "Unidentified" refers to mixtures of pigments not separated to permit calculation of yield of individual isomers.

** Figure represents maximum yield of isomer.

*** Figure represents minimum yield of pure isomer.

^Δ Dash indicates negligible quantity or that yield was not determined.

longer wave length maxima reported by Zechmeister and Polgár (2) are often 20 to 30 Å longer than the true wave lengths.

Approximate yields of the individual isomers obtained from different

isomers as starting materials are given in Table II. In many cases yields were calculated from experiments in which the primary purpose was the preparation of certain isomers in pure form. For this reason many of the figures are far from quantitative. The figures presented indicate the amount isolated quantitatively by several adsorptions, often with sacrifice of upper and lower parts of zones. Early in the work, however, it was noted that heat isomerization of some isomers yielded neo U, V, W as principal products and the heat isomerization of others yielded all-*trans* isomer as the principal product. Therefore, in all heat isomerization experiments particular attention was given to the relative yields of neo U, V, W and the all-*trans* form.

DISCUSSION OF RESULTS

Neo U, V, W

Separation of the isomers adsorbed above all-*trans* α -carotene into several zones, as accomplished by Zechmeister and Polgár (2), was not achieved by the writers. The difference may be due to the different adsorbents employed. The wave length shift from the all-*trans* isomer reported here for the neo U, V, W mixture lies between the shift (2) for the two principal isomers of the group, neo U and neo W. The total yield of neo U, V, W obtained from iodine-light isomerization by the writers is comparable to the sum of the yields of these isomers obtained by Zechmeister and Polgár (2). The absorption value reported here (Fig. 3) for the group at the *cis*-peak is somewhat lower than corresponding values reported by Zechmeister and Polgár (2) for neo U or W, the predominant members of the group.

It is to be noted by reference to Table II that the yield of the all-*trans* isomer obtained by heat isomerization of neo U, V, W was very low. Instead, 13% of the mixture was converted to neo X, which probably has a second double bond in the *cis*-position (2). The yield of the neo U, V, W group obtained by heat isomerization of the all-*trans* form at 65° was not above 3-4% of the starting material. When, however, the temperature of heat isomerization was increased to 99°, the yield of neo U, V, W was increased to 26%. The importance of the use of different temperatures in heat isomerization as a tool in indicating the relative stability of different double bonds toward *cis-trans* isomerism is thus clearly demonstrated.

Iodine-light isomerization of neo U, V, W gave rise to at least 7% of the all-*trans* isomer, confirming the ready interconvertibility of the all-

trans and neo U, V, W isomers by iodine-light. It was assumed without further study that the isomers which can be produced by iodine-light isomerization of the all-*trans* isomer itself were also present.

Neo X

As mentioned previously, neo X was obtained in appreciable yields only by heat isomerization of neo U, V, W at 65°. Reverse isomerization of this pigment by heat at 65° yielded only about 2.5% of the all-*trans* isomer and at least 53% of neo U, V, W. Thus the situation is analogous to that reported for methylbixin (1). Iodine-light isomerization or heat isomerization at higher temperatures is necessary to give large yields of neo U, V, W from the all-*trans* isomer. Neo U, V, W, once produced, can be converted to neo X more easily than to the all-*trans* isomer.

Neo B

This pigment was found just below the all-*trans* isomer on alumina columns. It was obtained in appreciable yields only by heat isomerization at 99° and by iodine-light isomerization; the yields in these cases being 18 and 20% of the starting material, respectively. Its reverse isomerization by heat at 65° yielded 20% of neo U, V, W and 30% of the all-*trans* form. The high yield of neo U, V, W indicates a close relationship of this isomer to at least one of the principal isomers of the neo U, V, W group.

Neo C

The isomer found by the authors to occupy the same position on a chromatogram as Zechmeister's neo C (2) showed a much greater displacement (80 Å) of the longer wave length maximum toward the shorter wave lengths than did Zechmeister's neo C (45 Å). However, since this isomer has the highest *cis*-peak of any of the neo- α -carotenes studied in this region (Fig. 3), and since the authors' preparation showed a higher *cis*-peak than Zechmeister's, it is possible that Zechmeister did not succeed in obtaining a preparation free of other isomers. Furthermore, Zechmeister and Polgár (6) have suggested that the double bond occupying the *cis*-position in neo C is one of the central double bonds, number 5 or 6. If, as hinted by Zechmeister (1), the double bonds near the center of the conjugated system cause relatively greater wave length shifts, then neo C would be expected to show a greater shift toward the shorter wavelengths than neo U or W, for example.

A mixed chromatogram showed neo C to be separable from neo B.

Neo C was obtained from the all-*trans* isomer by heat isomerization at 65° but was not obtained by heat isomerization at 99° or by iodine-light isomerization. This indicates a high degree of thermal lability. Reverse heat isomerization of this isomer gave the all-*trans* isomer as the principal product and only a very low yield of neo U, V, W.

Neo C'

This isomer was obtained in low yields from iodine-light isomerization mixtures and from heat isomerization of the all-*trans* isomer at 99°. Failure to obtain identical preparations from different experiments indicates a possibility that this isomer is an artifact resulting from a mixture of other isomers which were imperfectly separated on the column. It gave both the all-*trans* isomer and neo U, V, W by heat isomerization at 65°.

Neo D

Neo D was obtained by iodine-light isomerization and by heat isomerization at 99° but not at 65°. In addition to agreement in wavelength shifts, other evidence that this pigment corresponds to the neo D of Zechmeister (2) is the fact that he obtained relatively larger yields of this pigment by melting crystals at 195–200° than by other methods. This pigment is thus formed in larger amounts at higher temperatures.

Reverse isomerization of this pigment by heat at 65° yielded at least 19% neo U, V, W together with 33% of the all-*trans* isomer. This high yield of neo U, V, W probably indicates that one of the *cis*-bonds of neo D is the same one as that which occupies the *cis*-position in neo U or neo W.

Neo E

Neo E was obtained in 1.5% yield by heat isomerization. None was isolated from iodine-light isomerization mixtures, although Zechmeister (2) obtained about 1% by this method. Reverse heat isomerization at 65° yielded only 3.2% of neo U, V, W but considerable neo C (10%). Results of reverse heat isomerization (65°) of both neo C and E were similar except for the yield of all-*trans*, indicating some similar bond configuration.

SUMMARY

Members of the α -carotene stereoisomeric set obtained by heat and iodine-light isomerization were studied by chromatographic and spectroscopic techniques. Spectral absorption curves in the visible region are presented for the isomers, the coincident points of the curves with that of the all-*trans* isomer being established by isomerization methods. Ultraviolet data on the *cis*-peak were obtained for several isomers.

Relative yields of the various isomers were obtained by the two methods of isomerization, using different isomers as starting materials. The effect of temperature on yields of the isomers was shown to be important, particularly in the demonstration of relative stability of and structural relationships among certain isomers.

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The Apparent Dissociation Constants of Homocystine and Homocysteine

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Although homocystine and homocysteine are not considered as accepted amino acids since they have not been shown to be components of proteins, they nevertheless possess the characteristics of amino acids. In view of the numerous experiments that have been carried out on these compounds, it was deemed desirable to determine their apparent dissociation constants.

Both products were obtained from the S. M. A. Corporation. Purification was effected by dissolving with the aid of acid, precipitation by addition of alkali, and finally recrystallization from alcohol-water mixtures. Care was taken to prevent oxidation of homocysteine. The titration of the amino acids was carried out with the aid of a glass electrode. In alkaline solutions the Beckman "high pH" glass electrode was employed. The titrations were carried out in a cone shaped vessel similar to that described by Clark (1). The titration vessel was connected with the saturated calomel half cell by means of a saturated KCl bridge. The cell was kept in a 25° air bath. The pH measurements were made with the aid of a Goyan (2) amplifier and a Leeds and Northrup student potentiometer. The glass electrodes were standardized with a 0.05 *M* solution of potassium acid phthalate (pH 4.005) and a 0.05 *M* solution of sodium tetraborate (pH 9.18). The titration of homocysteine was carried out in an atmosphere of purified nitrogen in order to prevent oxidation. In view of the low solubility of homocystine, it was first dissolved in standard HCl and the solutions were brought to the various pH values by addition of known amounts of NaOH. The pH measurements were carried out quickly to avoid precipitation of the solute from the supersaturated solutions. No precipitation of homocystine was observed during the titrations.

It is apparent that homocysteine has one dissociation constant in the acid range and two overlapping dissociation constants in the basic range while homocystine possesses two pairs of overlapping constants, one pair in the acid range and one pair in the basic range. The acidic

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dissociation constant of homocysteine was derived separately from the relation

$$pK' = \text{pH} - \log \left(\frac{c}{a - [\text{H}^+]} - 1 \right)$$

where c denotes the concentration of the amino acid and a the concentration of HCl. The other regions of the titration curves were treated as mixtures of univalent weak acids having constants $G_1, G_2 \dots$. These constants were obtained by the method of Simms (3) which involves successive approximations. The experimental pH values were plotted against b' "corrected equivalents of base" and the pG' values were estimated graphically from the curves. At each pH within the region of a given pG' value the α values corresponding to the other pG' values were calculated. Subtracting these from b' the values of the desired pG' were obtained. These were inserted in the mass law expression

$$pG' = \text{pH} - \log \frac{\alpha}{1 - \alpha}$$

in order to obtain values for pG' . The titration constants were corrected with the aid of the equations

$$K'_1 K'_2 = G'_1 G'_2 \text{ and } K'_1 = G'_1 + G'_2$$

and values for the apparent dissociation constants were thus obtained. The following are the values for homocystine: $pK'_1 = 1.59$; $pK'_2 = 2.54$; $pK'_3 = 8.52$; $pK'_4 = 9.44$. The values for homocysteine are: $pK'_1 = 2.22$; $pK'_2 = 8.87$; $pK'_3 = 10.86$. These values are slightly greater than the corresponding values for cystine and cysteine (4) and are due to the greater length of the amino acid chains.

The titration curves of homocystine and homocysteine are represented in Figs. 1 and 2 respectively. The points are experimental and the curves are theoretical. The latter are based on the constants given in Tables I and II.

They were plotted with the aid of the equation

$$b' = \frac{b - a}{c} + \frac{[\text{H}^+] - [\text{OH}^-]}{c} = \frac{2 + \frac{[\text{H}^+]}{K'_2}}{1 + \frac{[\text{H}^+]}{K'_2} + \frac{[\text{H}^+]^2}{K'_1 K'_2}}$$

where b' = "corrected equivalents of base," b = molal concentration of strong base, a = molal concentration of strong acid, c = molal concen-

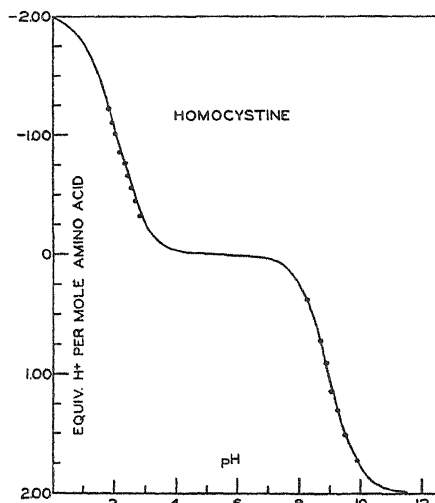


FIG. 1

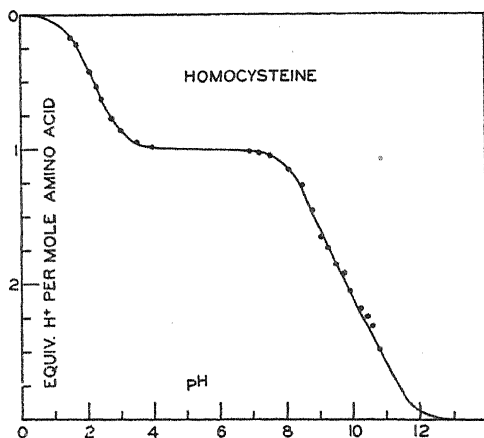


FIG. 2

tration of substance, and $K'_1K'_2$ = dissociation constants of polyvalent acid representing steps in the ionization of the amino acid.

TABLE I
Titration of Homocystine at 25°
(a) Acid solutions¹

pH	$\frac{a-b}{c}$	$\frac{[H^+] - [OH^-]}{c}$	$-b'$	$-b' - (\alpha)_2$	$-b' - (\alpha)_1$	pG'_1	pG'_2
1.861	2.969	1.742	1.227	0.405		1.694	
1.952	2.513	1.413	1.100	0.311		1.607	
2.085	2.047	1.040	1.007	0.274		1.662	
2.212	1.628	0.776	0.852		0.635	(1.654) ²	2.452
2.365	1.308	0.546	0.762		0.599		2.539
2.415	1.195	0.486	0.709		0.561		2.521
2.459	1.105	0.439	0.666		0.531		2.513
2.508	1.028	0.393	0.635		0.512		2.529
2.556	0.911	0.352	0.559		0.448		2.465
2.632	0.798	0.295	0.503		0.408		2.471
2.682	0.709	0.263	0.446		0.360		2.433
2.770	0.594	0.215	0.379		0.308		2.418
2.835	0.506	0.185	0.321		0.259		2.379
							(2.476) ²

(b) Alkaline solutions³

pH	$\frac{b-a}{c}$	$\frac{[H^+] - [OH^-]}{c}$	b'	$b' - (\alpha)_4$	$b' - (\alpha)_3$	pG'_3	pG'_4
8.271	0.381		0.381	0.307		8.624	
8.602	0.691		0.691	0.545		8.524	
8.715	0.720		0.720	0.539		8.647	
8.878	0.918	0.001	0.917	0.673		8.565	
9.094	1.144	0.001	1.143		0.382	(8.590) ²	9.303
9.134	1.174	0.001	1.173		0.395		9.319
9.313	1.303	0.002	1.301		0.460		9.382
9.423	1.307	0.003	1.367		0.495		9.432
9.565	1.511	0.004	1.507		0.603		9.383
9.913	1.728	0.009	1.719		0.764		9.401
							(9.370) ²

¹ Concentration of amino acid, $9.036 \times 10^{-3} M$ (constant); conc. of HCl, 0.0970 M ; $-\log \gamma_{HCl}$ was assumed to be 0.058 (Lewis and Randall).

² The values given in parentheses are the average pG' values.

³ Concentration of amino acid, $9.036 \times 10^{-3} M$ (constant); conc. of NaOH, 0.1029 M ; $-\log \gamma_{NaOH}$ was assumed to be 1.000; $-\log Kw$ was assumed to be 13.996 (Harned).

Av. pK' values: $pK'_1 = 1.593$; $pK'_2 = 2.537$; $pK'_3 = 8.523$; $pK'_4 = 9.437$. These values were obtained from the corresponding pG' values by the use of appropriate equations.

TABLE II
Titration of Homocysteine at 25°

(a) Acid solutions¹

pH	γ_{HCl}	$-\log [\text{H}^+]$	$a \times 10^4$	$c \times 10^2$	$\frac{a - [\text{H}^+]}{c}$	$\frac{\log c}{a - [\text{H}^+]}$	pK'_1
3.962	0.992	3.959	3.904	1.494	0.0188	1.718	2.244
3.642	0.987	3.636	7.784	1.489	0.0367	1.419	2.223
3.471	0.982	3.463	11.61	1.482	0.0551	1.234	2.237
3.339	0.979	3.330	15.43	1.477	0.0728	1.105	2.234
3.221	0.974	3.210	19.22	1.471	0.0887	1.012	2.209
3.132	0.969	3.118	22.96	1.464	0.1047	0.932	2.200
3.060	0.966	3.045	26.68	1.459	0.1211	0.861	2.199
3.001	0.963	2.985	30.38	1.453	0.1378	0.796	2.205
2.887	0.956	2.868	37.68	1.442	0.1673	0.697	2.190
2.816	0.950	2.794	44.89	1.431	0.2014	0.598	2.218
2.734	0.946	2.710	52.00	1.420	0.2289	0.527	2.207
2.643	0.940	2.616	62.42	1.404	0.2722	0.427	2.216
2.557	0.935	2.528	72.71	1.388	0.3102	0.347	2.210
2.448	0.928	2.416	89.19	1.363	0.3729	0.226	2.222
2.268	0.916	2.230	120.5	1.314	0.4689	0.0539	2.214
2.084	0.903	2.044	163.4	1.248	0.5785	0.1370	2.221
1.679	0.877	1.622	317.8	1.011	0.7815	0.5540	2.233
1.506	0.865	1.443	430.5	0.8371	0.8350	0.7030	2.209
							(2.222) ²
4.043	0.993	4.040	3.193	1.488	0.0157	1.808	2.235
3.858	0.991	3.854	4.771	1.482	0.0227	1.633	2.225
3.743	0.984	3.738	6.337	1.477	0.0305	1.502	2.241
3.649	0.987	3.643	7.891	1.471	0.0382	1.401	2.248
3.492	0.983	3.485	10.95	1.459	0.0526	1.255	2.237
3.427	0.981	3.419	12.48	1.453	0.0596	1.198	2.229
3.322	0.978	3.312	15.47	1.442	0.0735	1.100	2.222
3.194	0.972	3.182	19.90	1.425	0.0935	0.987	2.207
3.070	0.966	3.055	25.63	1.404	0.1198	0.866	2.204
2.938	0.960	2.920	32.58	1.378	0.1492	0.756	2.182
2.801	0.951	2.779	43.14	1.339	0.1981	0.607	2.194
2.493	0.932	2.462	77.89	1.208	0.3591	0.251	2.242
							(2.222) ²

¹ Initial concentration of amino acid, 0.015 *M*. For that portion of the table corresponding to pH 3.962–1.506 the concentration of HCl used was 0.0974 *M* while for pH 4.043–2.493 it was 0.040 *M*. Standard for glass electrode, 0.05 *M* potassium acid phthalate = pH 4.005, saturated KCl junction.

² The values given in parentheses are average values.

[illegible]

⁴ The heading of the column for the values that follow is $b \times 10^3$.
Av. pK' values: $pK'_1 = 2.222$; $pK'_2 = 8.87$; $pK'_3 = 10.86$.

DISCUSSION

In the case of homocysteine pK'_1 may be assigned without hesitation to the carboxyl group. The influence of the NH_3^+ group and the sulfhydryl group is acid strengthening. The difference in acid strength of the various thio acids depends on the substituent groups and the distance of the sulfhydryl group from the carboxyl group. It is possible to calculate the strength of these acids with a fair degree of accuracy by considering them as derivatives of acetic acid. Using the principle of Derick (5) and neglecting steric hindrance and solvation effects, the dissociation constant of a fatty acid derivative is given by the equation

$$pK = 4.75 - \frac{A_\alpha \sum I\alpha^{i_\alpha}}{1 + [B_\alpha \sum I\alpha^{i_\alpha}]} - \log \frac{n'}{m'}$$

TABLE III

Apparent Dissociation Constants of Certain Thio Acids

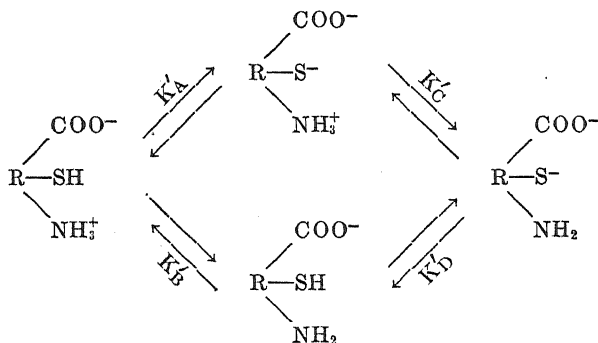
Acid	pK'_1 (obs.)	pK'_1 (calc.)
α -Thiobutyric	3.7	3.9
Thioglycolic	3.5	3.8
α -Thiolactic	3.7	3.9
β -Thiolactic	4.3	4.5
Cysteine	2.0	1.7
Homocysteine	2.2	1.9

where A_α and B_α are constants corresponding to the values 0.3 and 0.03 respectively, n' and m' are the numbers of equivalent carboxyl and carboxylate groups, I is the inductive constant for each atom other than hydrogen and for a formal charge, α is the fraction that reduces the inductive effect for each transmission across an atom, and i_α is the number of atoms intervening between a substituent group and the α -carbon atom of a carboxylic acid. The values for I , α , A_α , and B_α have been taken from Branch and Calvin (6). Table III gives the calculated and observed pK' values for a number of thio acids. The calculated and observed values agree within 0.3 pK' . A more exact correlation would not be expected in view of these assumptions and simplifications made.

The allocation of pK'_2 to the sulfhydryl or the amino group presents some difficulties. The allocation cannot be made on the basis of the titration data alone. The values for pK'_2 and pK'_3 are small. They represent composite constants in that the dissociation of one group is af-

fect by that of the other. A comparison of the dissociation constants of β -alanine ($pK'_2 = 10.3$) and β -thiolactic acid ($pK'_2 = 10.5$) lends support to the view that the acidic strength of the sulfhydryl group is almost equal to that of the amino group. It appears reasonable to assume that the values of pK'_2 and pK'_3 of homocysteine are determined almost to an equal extent by the dissociation of the ammonium and sulfhydryl groups. The same conclusion, as regards cysteine, was drawn by Cannan and Knight (7).

Edsall (8) has pointed out that the observed values of pK'_2 and pK'_3 , for a thioamino acid, may be expressed in terms of the following steps for the dissociation of the individual groups:



These constants are related as follows to the measured pK'_2 and pK'_3 values¹:

$$K'_2 = K'_A + K'_B; \quad \frac{1}{K'_3} = \frac{1}{K'_C} + \frac{1}{K'_D}$$

$$K'_2 K'_3 = K'_A K'_C = K'_B K'_D$$

He points out that the $-\text{SCH}_3$ or $-\text{SC}_2\text{H}_5$ group is probably very similar to the $-\text{SH}$ group in the effect on the dissociation of a neighboring group. On this assumption, pK'_B for cysteine should be very close to pK'_2 for *S*-ethyleysteine, which Ratner and Clarke (12) have found to be 8.60. Likewise pK'_B for homocysteine should be nearly equal to pK'_2 for

¹ The argument is identical with that employed in calculating the ratio of dipolar ions to unchanged molecules from the pK' values of the amino acids and their esters. See Ebert (9), Miyamoto and Schmidt (10), and Edsall and Blanchard (11).

methionine (*S*-methylhomocysteine) which Emerson, Kirk, and Schmidt (13) found to be 9.21. Using the values of pK'_2 and pK'_3 for cysteine reported by Borsook, Ellis, and Huffman (14), and those for homocysteine reported here, the following values of the various constants are obtained:

Amino acid.....	pK'_2	pK'_3	pK'_A	pK'_B	pK'_C	pK'_D
Cysteine.....	8.33	10.78	8.66	8.60	10.45	10.51
Homocysteine....	8.87	10.86	9.14	9.21	10.59	10.52

Thus the pK' values of the $-\text{SH}$ group and the $-\text{NH}_3^+$ group, under comparable conditions, are very nearly the same. The closer together the two groups are in the molecule, the greater is their interaction, which results in increasing the acidity of both groups.

We are indebted to Dr. John T. Edsall for valuable suggestions in the preparation of the manuscript.

SUMMARY

1. The apparent dissociation constants for homocystine were determined to be 1.59, 2.54, 8.52, and 9.44 and for homocysteine 2.22, 8.87, and 10.86.

2. Comparison has been made between the pK' values of certain thioacids calculated with the aid of the modified Derick equation and those obtained experimentally.

3. In the case of homocysteine and other sulfhydryl amino acids assignment of the pK'_2 and pK'_3 values to specific groups cannot be made due to the fact that they represent composite constants. The intrinsic acidity of the sulfhydryl group is close to that of the ammonium group. The acidity of each group is strengthened by the presence of the other in the molecule.

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The Activity of Penicillin Combined With Other Anti-Streptococcal Agents Towards β -Hemolytic Streptococci in Vivo

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Penicillin exerts a remarkably high activity on infections caused by β -hemolytic streptococci in mice (1-4). Extensive work on the activity of penicillin *in vivo* carried out in this laboratory (5) during recent years has established the fact that a single subcutaneous injection of 6 to 10 Oxford units was sufficient to control an intra-abdominal infection caused by 1000 to 10,000 times the minimal lethal dose of β -hemolytic streptococci. Penicillin preparations of different degrees of purity and accordingly of different potency, as indicated by the cup test *in vitro*, have been used in these experiments in which nine strains of *Streptococcus hemolyticus* of Types 1, 2, and 3 (Group A) were tested.

In connection with these studies on the standardization of penicillin *in vivo*, experiments were undertaken in order to investigate more closely the synergistic activity of penicillin and sulfapyridine *in vivo*. Ungar, who recently described this synergistic activity (6), showed that a total dose of 144 units of a sodium penicillate which was obviously very low in anti-streptococcal activity *in vivo* was activated by the simultaneous intra-abdominal administration of a subtherapeutic dose of sulfapyridine (a total of 4 mg.). After the injection of either penicillin or sulfapyridine, only one mouse of six mice survived the fatal infection with streptococci, while the combination of both drugs protected four animals out of six.

In our first group of experiments the combined administration of penicillin and sulfonamides (sulfanilamide, sulfapyridine) was investigated. Experiments in which penicillin was given together with *p*-aminobenzoic acid and *p*-nitrobenzoic acid were also included. The former was selected because it potentiates the activity of penicillin *in vitro*, according to Ungar (6); the latter was tested on account of its slight anti-streptococcal activity (7-9).

In a second group of experiments penicillin was combined with two acridine dyestuffs (acriflavine and 2-nitro-5(γ -diethylamino- β -hydroxy-propylamino)-7,8-dimethoxyacridine¹ (10)) and with myochrysine, a gold compound of good anti-streptococcal activity (11).

The third group of experiments dealt with the influence of *p*-aminobenzoic acid on the combined activity of penicillin and sulfanilamide.

Methods—The experiments were carried out on white mice weighing 18 to 22 g. Groups of at least ten animals were used.

Streptococcus Strain—A β -hemolytic streptococcus Strain 4 (Group A, Type 3) was used in all experiments. The strain was passed through mice regularly and possessed a high virulence for them. The minimal lethal dose which killed 50% of the infected mice within 36 to 48 hours was 0.5 cc. of a 10^{-8} to 10^{-9} dilution of an overnight culture on serum broth, injected intra-abdominally. The infection used in these experiments was 0.5 cc. of a 10^{-6} dilution.

Treatment—The treatment was adapted to the standard procedures used in this laboratory. A single subcutaneous injection of penicillin was made immediately after the infection. Penicillin "CS" used in these experiments was a sodium salt prepared by the chemical research laboratories. Its potency as determined by the cup test with *Staphylococcus aureus* was 100 units per mg. The dose used in most of these experiments was 0.02 and 0.03 mg. (2 to 3 units) per 20 g. of mouse, representing one-third to one-fifth of the minimal active dose.

The sulfonamides as well as *p*-amino- and *p*-nitrobenzoic acid were given repeatedly *per os* suspended in 10% gum arabic. The first dose was administered immediately after the infection; the second, 6 hours later. On the following day, two more doses were given with a 7 to 8 hour interval, and one more dose on each of the 2 following days. The following subtherapeutic doses were used: sulfanilamide, 0.5 mg. = one half of the minimal active dose; sulfapyridine, 0.5 to 0.3 mg. = one-half to one third of the minimal active dose; *p*-nitrobenzoic acid, 5 mg. = one half of the minimal active dose; *p*-aminobenzoic acid, 25 mg. = one half of the tolerated (inactive) dose.

In the second group of experiments, the same penicillin was used in the same way as described before. The acridine dyestuffs as well as the gold compound were administered also subcutaneously but remote from the site of the injection of penicillin, shortly after the infection. The following doses were used: gonaerine (= acriflavine = 2,8-diaminoacridine-10-N-methochloride), 0.3 mg. = the tolerated dose; nitroacridine compound, 0.5 mg. = one fourth of the minimal active dose; myochrysine (Na gold thiomalate (Merck)), 0.3 to 0.5 mg. = one sixth to one fourth of the minimal active dose.

In the experiments in which penicillin and sulfanilamide were combined with *p*-aminobenzoic acid, the same technique was used with slight modifications. Penicillin (0.03 mg.) was given subcutaneously shortly after the infection. Only one oral treatment with a mixture of 2 to 5 mg. of sulfanilamide + 10 to 20 mg. of *p*-aminobenzoic acid, suspended in 10% gum arabic, was given at the same time.

¹ Marked "nitroacridine compound" in Table II.

All doses were adapted to 20 g. of body weight. Controls treated with penicillin alone and with the other drugs alone were included in the experiments as well as untreated controls. The surviving mice were observed over a period of 20 days.

TABLE I

Combination of Subtherapeutic Doses of Penicillin with Sulfanilamide, Sulfapyridine, p-Nitrobenzoic Acid, and p-Aminobenzoic Acid in Experimental Streptococcus Infections in Mice

Drug	Dose* mg.	Penicillin dose† mg.	No. of mice	No. of survivors	Survivors per cent
Sulfanilamide	0.5		10	4	40
	0.5	0.02	10	10	100
		0.02	10	0	0
	0.1		20	1	5
	0.1	0.03	20	5	25
		0.03	20	1	5
Sulfapyridine	2		10	5	50
	2	0.02	10	10	100
		0.02	10	0	0
	0.5		10	0	0
	0.5	0.02	10	9	90
		0.02	10	1	10
	0.3		10	2	20
	0.3	0.02	10	8	80
		0.02	10	2	20
p-Nitrobenzoic acid	5		20	3	15
	5	0.02	18	14	78
		0.02	20	3	15
p-Aminobenzoic acid.....	25		10	0	0
	25	0.02	10	0	0
		0.02	10	2	2
Controls			65	0	0

* Six times repeated oral treatment.

† One single subcutaneous treatment.

EXPERIMENTAL

1. Combination of Penicillin with Sulfanilamide, Sulfapyridine, or p-Aminobenzoic Acid

The results of these experiments, given in Table I, confirmed Ungar's observation of the synergistic activity of subtherapeutic doses of penicillin and sulfapyridine. A marked effect was also obtained with

penicillin + sulfanilamide and with penicillin + *p*-nitrobenzoic acid, but not with penicillin + *p*-aminobenzoic acid. Very small doses of sulfapyridine, such as 0.5 to 0.3 mg. per 20 g., which were practically inactive still gave 80 to 90% of survivors if combined with a low dose of penicillin. The experiments with sulfanilamide demonstrated that the synergistic effect was dependent on the dose. While a moderately low dose, such as 0.5 mg. per 20 g., protected 100% of the infected mice when given together with 0.02 mg. of penicillin, no significant effect was seen when 0.1 mg. of sulfanilamide was combined with 0.03 mg. of penicillin. *p*-Aminobenzoic acid, which lacks anti-streptococcal activity *in vivo*, failed to show a synergistic activity with penicillin. The slight anti-streptococcal activity of *p*-nitrobenzoic acid was, however, considerably enhanced. The question arose whether other chemotherapeutic agents which exert an anti-streptococcal activity might show synergism with penicillin.

2. Combination of Penicillin with Acridine Dyestuffs and Myochrysine

That no synergistic activity could be found when penicillin was given in combination with anti-streptococcal agents of the acridine group or with a gold compound is shown in Table II.

This striking difference as to the synergistic activity of sulfonamides + penicillin on one hand, and other anti-streptococcal agents on the other, suggested a more intense study of the mechanism of the synergism. So far, the rôle of the sulfonamides in this combination has been investigated.

3. Combination of Penicillin and Mixtures of Sulfanilamide and *p*-Aminobenzoic Acid

The experiments described in the first part of this paper showed that the success of the combined dosage with penicillin and sulfanilamide was more or less dependent on the dose of the sulfonamide. In order to reduce the activity of the sulfonamide completely, penicillin was combined with a mixture of larger doses of sulfanilamide and *p*-aminobenzoic acid. There was no indication that the activity of penicillin would be affected by *p*-aminobenzoic acid. It is known (Fleming (12)) that there exists no antagonism of *p*-aminobenzoic acid to penicillin, nor is a synergism of the two agents evident *in vivo* (Table I).

Table III demonstrates that *p*-aminobenzoic acid, though it inhibits the activity of sulfanilamide almost completely (2 survivors out of a

TABLE II

Combination of Subtherapeutic Doses of Penicillin with Acridine Dye-stuffs and Gold Compounds in Experimental Streptococcus Infection in Mice

Drug	Dose*	Penicillin*	No. of mice	No. of survivors
	mg.	mg.		
Acriflavine	0.3		10	0
	0.3	0.02	10	0
		0.02	10	0
Nitroacridine compound	0.5†		10	1
	0.5†	0.02	10	1
		0.02	10	0
Myochrysine	0.3		10	0
	0.3	0.02	10	0
		0.02	5	0
“	0.5		10	0
	0.5	0.033	10	0
		0.033	10	0
Controls			40	1

* Subcutaneous treatment.

† Total of four treatments, 2 mg.

TABLE III

Activity of Penicillin CS and Sulfanilamide in Presence of p-Aminobenzoic Acid

SA = sulfanilamide; PABA = p-aminobenzoic acid; P = penicillin; C = untreated controls.

Dose		No. of survivors out of 10 after treatment with					
SA	PABA	P	SA	SA + PABA	P + SA	P + SA + PABA	C
mg.	mg.						
5	10	4	6	1		8	0
5	10	2	0	1		6	0
5	10	1	3	0		8	1
5	10	0	0	0	4	4	0
2	10	2	1	0	4	2	0
2	10	0	0	0	2	0	0
4	20			0		4	
2	20			0		0	

total of 80 mice = 2.5%), does not, to the same extent, interfere with the synergistic system of penicillin + sulfanilamide. With the com-

bined administration of penicillin + sulfanilamide + *p*-aminobenzoic acid a total of 32 survivors out of 80 mice (= 40%) was observed. This corresponds closely to the results obtained with penicillin + sulfanilamide (33%²), while penicillin alone and the comparatively high doses of sulfanilamide alone protected only 15 to 16% of the mice.

These percentage figures were computed regardless of the dose of sulfanilamide and *p*-aminobenzoic acid. A comparison of the results according to the doses used in the different experiments (Table IV) indicates that the success of the combination depends on the amount of sulfanilamide available.

If a constant, small dose of penicillin was combined with decreasing doses of sulfanilamide plus increasing doses of *p*-aminobenzoic acid, the activity of the combined drugs dropped gradually to zero.

TABLE IV

Activity of Penicillin CS + Sulfanilamide in Presence of p-Aminobenzoic Acid
(condensed from Table III)

Penicillin CS, 30 γ plus	No. of mice	No. of survivors	Survivors per cent
5 mg. sulfanilamide + 10 mg. <i>p</i> -aminobenzoic acid.....	40	26	65
4 mg. sulfanilamide + 20 mg. <i>p</i> -aminobenzoic acid.....	10	4	40
2 mg. sulfanilamide + 10 mg. <i>p</i> -aminobenzoic acid.....	20	2	10
2 mg. sulfanilamide + 20 mg. <i>p</i> -aminobenzoic acid.....	10	0	0

DISCUSSION

The experiments presented in this paper confirm Ungar's findings as to the synergistic activity of penicillin and sulfapyridine in experimental streptococcus infections of mice. A similarly good effect is obtained when penicillin and sulfanilamide or penicillin and *p*-nitrobenzoic acid are given. Very small amounts of sulfanilamide seemed sufficient for successful combination with small doses of penicillin. The minimal dose of sulfanilamide active under these experimental conditions was found in the range between 0.5 and 0.1 mg. administered repeatedly *per os*. The experiments in which penicillin was combined with sulfanilamide

² The results of these experiments are less striking than of those described in Part I, because only *one single* treatment with sulfanilamide was given.

+ *p*-aminobenzoic acid confirmed these results. Penicillin counteracted the inhibiting action exerted by *p*-aminobenzoic acid on sulfanilamide. Here, too, the effect of the combination was dependent on the dose of sulfanilamide and the inhibitor; if the dose of the sulfonamide decreased and the dose of the inhibitor increased, the therapeutic activity of the combined system dropped significantly. These observations seem to substantiate the view that the combined effect of penicillin and sulfonamides should be interpreted as a synergism of the two different agents. Ungar (6) mentioned the possibility, though as a remote one, that a new active reaction product of penicillin and sulfonamides might be formed in the organism of the host, but no evidence for such a reaction could be offered on the basis of his or our experiments.

We are inclined to assume that a different mechanism of activity of penicillin on one side, and of sulfonamides on the other, facilitates this synergism. The mechanism which determines the activity of acridine dyestuffs and gold compounds appears to be definitely different from that of the sulfonamides and prevents a successful combination of these drugs with penicillin. This question warrants further investigation, and the use of drug-resistant strains seems to offer a promising approach to its solution.

SUMMARY

1. A single subcutaneous injection of a subtherapeutic dose of sodium penicillate (2 to 3 units) was combined with equally small doses of sulfonamides and related compounds as well as with two different anti-streptococcal acridine dyestuffs and a gold compound (myochrysine) in experimental streptococcus infections of white mice.

2. In confirmation of the findings of Ungar, it was found that small doses of penicillin have a striking synergistic effect on the activity of sulfapyridine. An equally good effect was observed if penicillin was given together with sulfanilamide or *p*-nitrobenzoic acid.

3. No activity was found when penicillin was combined with *p*-aminobenzoic acid.

4. Penicillin did not show a combined effect with acriflavine, 2-nitro-5(γ -diethylamino- β -hydroxypropylamino-7,8-dimethoxyacridine, or with myochrysine.

5. Penicillin counteracts the inhibiting activity exerted by *p*-aminobenzoic acid on sulfanilamide.

6. Ungar's synergistic phenomenon is dependent on the amount of

sulfanilamide. If the dose of the sulfonamide is too small or if the dose of the inhibitor is too high, the activity of the synergistic system drops gradually to zero.

7. No evidence was found which would indicate the formation of an active reaction product of sulfonamides and penicillin.

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Stereochemical Configuration and Provitamin A Activity

I. All-*trans*- β -Carotene and Neo- β -Carotene U

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INTRODUCTION

While certain structural features of carotenoids have been determined, which in a positive or negative sense influence the provitamin A effect, not much is known about the relationship between stereochemical configuration and biological activity.

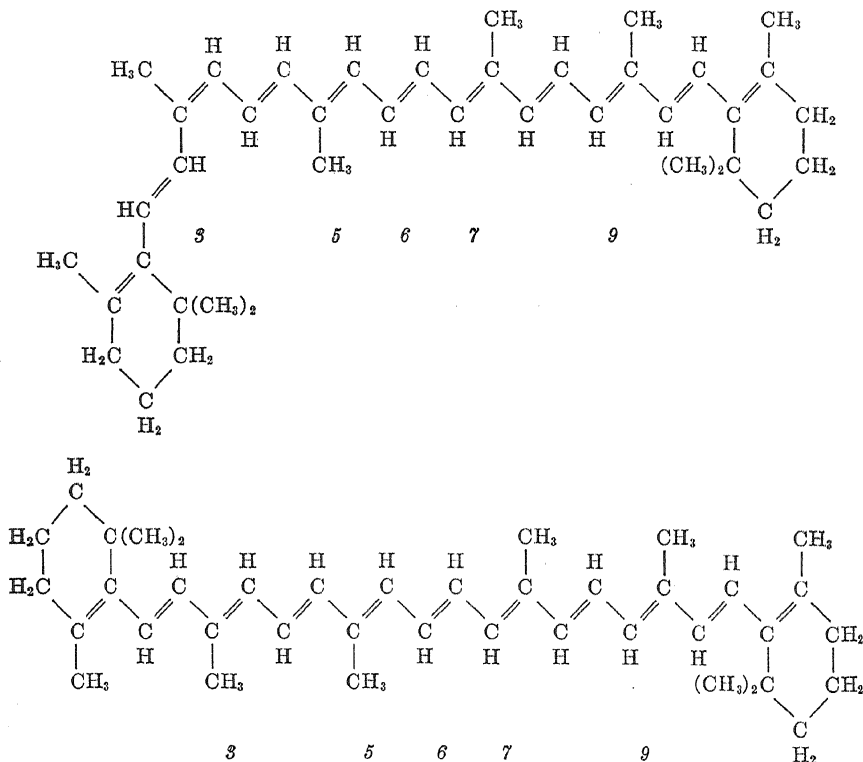
The chromophoric system of carotenoids is subject to *trans*→*cis* rearrangements, which have been studied by several authors and recently discussed in a monograph (11).

The first statement about the effect of configuration was made by Gillam and El Ridi (3) and refers to an isomer of β -carotene which is adsorbed below the natural pigment on the Tswett column and was termed "pseudo- α -carotene" (neo- β -carotene B). The authors mentioned the effectiveness of this substance in the rat and stated later, in collaboration with Kon (4), that "the vitamin A potencies of pseudo- α -carotene and β -carotene are of the same order." The same isomer, under the designation of "neo- β -carotene", was also tested by Kemmerer and Fraps (2, 8) who mentioned that this compound "has one-half the potency of β -carotene."

"Carotenoid X" which was obtained by Kemmerer and Fraps from plant extracts and which is chromatographically and spectroscopically identical with neo- β -carotene U, was found by these investigators to be inactive in the rat.

Results concerning the vitamin A activity of isomerized α -carotene, "neo- α -carotene" and "neocarotene", were reported by Gillam, El Ridi, and Kon (4). Furthermore, a neocryptoxanthin was investigated by Fraps and Kemmerer (8).

Two years ago, the isomerization of β -carotene was investigated more in detail (10) and it was shown that under suitable conditions about a dozen isomers can be observed in the chromatographic column. One of these members of the stereoisomeric β -carotene set, *viz.*, neo- β -carotene U (which is adsorbed above the β -carotene zone), possesses a remarkable degree of stability and was isolated in crystals which could be kept for several months, in the absence of air, without change of configuration. On the basis of a theoretical discussion given in collaboration with Pauling, LeRosen, and Schroeder (12) it was recently pointed out (13) that the neo- β -carotene U molecule contains one double bond in *cis* configuration and that this double bond is located near the end of the chromophoric system (see formulas below).



Above: Neo- β -carotene U = β -mono-*cis*- β -carotene.

Below: Natural β -carotene = all-*trans*- β -carotene.

(The double bonds which are sterically not hindered and able to perform *cis-trans* rotations are numbered.)

In preliminary tests carried out by Dr. C. E. P. Jeffreys and independently in the Research Laboratory of Merck & Co., Inc., it was found that neo- β -carotene U shows some provitamin A effect which is, however, weaker than that of β -carotene.¹

In the present paper we intend to report the results of a detailed study concerning the relative provitamin A efficiencies of natural (all-*trans*-) β -carotene and neo- β -carotene U in the rat.

EXPERIMENTAL

Materials

Neo- β -carotene U was isolated as described earlier (10). The freshly prepared Wesson oil (refined cottonseed oil) was obtained directly from the Southern Cotton Oil Co., Savannah, Ga. Its color was slightly yellow which, however, as shown by spectroscopy, was not caused by carotenoids. The oil showed transmission down to 330 m μ in the Beckman spectrophotometer. Upon saponification of a petroleum ether solution with methanolic potassium hydroxide at 25° a dark color appeared which could be washed out with water. The remaining petroleum ether solution was practically colorless.

In order to test the stability of neo- β -carotene U in the original Wesson oil, a solution of 1 mg. of crystals in 1 ml. of the oil containing 6 mg. of α -tocopherol was kept under carbon dioxide at 4 to 5° for three days, and then saponified. A chromatogram revealed that 6.5% of the total recovered pigment was all-*trans*- β -carotene, formed by re-isomerization during all operations. After the conclusion of the bioassays described below, a sample of the original neo- β -carotene U crystals proved to be practically homogeneous in a chromatogram.

Bioassays

The bioassays were made according to the U.S.P. XII methods with the exception that the depletion period was 17-18 days in most cases instead of 20 days. Two sources of the U.S.P. XII diet were employed, namely, a commercially prepared vitamin A test diet (obtained from S.M.A., Chagrin Falls, Ohio) and one prepared in our laboratory. In the latter case the fat used was coconut oil containing 1 mg. of α -tocopherol per gram of oil. Approximately two-thirds of the rats in each group received the first diet and one-third the second. Identical results were obtained in both diets. The rats employed in the test were obtained from Sprague-Dawley, Inc., Madison, Wis.

The supplements were administered daily² instead of six times weekly. They were made fresh every third day from approximately 0.5 mg. samples of β -carotene and neo- β -carotene U crystals prepared shortly before the start of the test and kept in tubes sealed under CO₂ in the refrigerator until used.

¹ The statement in our paper (10) that no activity has been found was caused by a misprint and was later corrected; cf. *J. Am. Chem. Soc.* **64**, 3071 (1942).

² The supplement was administered on the first Sunday and on the following three weekends at 1.5 times the daily dose on Saturday and Monday, and no dose on these Sundays.

The oil solutions of the carotenes were prepared by grinding the material in a dark room on an agate mortar with approximately 10 ml. of Wesson oil containing 5 mg. of added α -tocopherol (Merek & Co.) per ml. The liquids were centrifuged and the potency determined on the clear oil solution, on the Klett-Sumner photoelectric colorimeter using the "420 m μ filter," by a modification of the method of Koehn and Sherman (9). Corrections which amounted to less than 3% were made for the non-carotenoid color due to similar amounts of Wesson oil.

Supplements were made up by weighing appropriate amounts of the pigment solutions and diluting to volume in 10 ml. volumetric flasks of actinic glassware with Wesson oil containing 5 mg. of α -tocopherol per ml. This gives a concentration of 0.5 mg. tocopherol per 0.1 ml. which Hickman *et al.* (5) have found is the quantity required for the optimum utilization of β -carotene. The supplements were kept under CO₂ in small amber bottles in the refrigerator.

After the animals had exhibited symptoms of vitamin A deficiency, such as constant weight over 5 days and xerophthalmia, they were assigned to one of seven groups. Six of these groups consisted of 16 rats each (including both sexes) and the seventh was made up of 7 females. These each received one of the following supplements daily in 0.1 ml. of Wesson oil containing 0.5 mg. of α -tocopherol: oil-tocopherol alone (negative control group); 0.9 or 2.1 μ g. of β -carotene; 0.9, 2.1, 3.3, or 9.0 μ g. of neo- β -carotene U.

RESULTS

Table I gives a summary of the data obtained on the 103 rats used. To avoid small discrepancies due to uneven distribution of the sexes in the various tests, the data for the males and females are weighted equally. The accuracy in the averages is indicated by the uniformity in the gains in weight in individual rats which are given in Table II.

The results of the negative controls indicate that the rats were sufficiently depleted in vitamin A at the start of the test and that the basal diet was essentially vitamin A-free. The eye symptoms which were present in all rats before starting on the assay period were promptly cleared up in the groups receiving both levels of β -carotene and the three highest levels of neo- β -carotene U.

The dosage/gain in weight curves for β -carotene and neo- β -carotene are given in Fig. 1. The fact that the three points on the curve for neo- β -carotene U fall on a straight line is a proof of the accuracy of the results with neo- β -carotene U as well as a confirmation of the concept of this relationship of the vitamin A between growth and dosage. The accuracy of all data is also suggested by the fact that the two curves are reasonably parallel. The potency of the neo- β -carotene U was calculated by the method of Coward (1) as 2.6 and 2.7 μ g. being biologically equivalent to 1.0 μ g. of β -carotene. These values were obtained as follows: The projection of the average gain in weight of rats receiving

TABLE I
*Summary Table of Bioassay Experiments on Male and Female Rats Receiving β -Carotene or Neo- β -carotene U, in Cottonseed Oil or the Oil Alone (Average Results on Males and Females Weighted Equally)**

Supplement	Dose per day	Number of rats	Depletion period			Final weight	Increase in body weight on following days					Assay period			Final weight
			Male	Female	Start		Age	Weight	Length	5th	10th	15th	20th	25th	
	μ g.		days	g.	days	g.	days	g.	g.	g.	g.	g.	g.	g.	g.
Negative controls	con-	0.0	7	9	28.0	44.4	18.2	74.8	-0.8	0.0	-1.4 (13)	-10.3 (11)	-13.0 (3)	-18.0 (2)	56.5 (2)
β -Carotene	0.9	8	27.6	43.4	18.4	75.4	6.6	18.3	29.2	38.2	47.8	53.6	129.1		
	2.1	7	27.7	44.0	18.2	75.1	11.7	28.7	40.8	53.1	67.1	74.7	148.9		
Neo- β -carotene U	0.9	6	27.7	43.2	18.2	74.0	0.9	6.0	11.4 (15)	15.2 (15)	19.6 (14)	22.0 (13)	94.8		
	2.1	7	27.8	43.2	18.2	72.8	5.9	16.2	23.6	33.6	45.1	49.6	122.4		
	3.3	8	27.8	44.8	18.2	76.9	5.5	20.1	32.5	42.1	55.5	61.9	138.8		
	9.0	0	28.0	44.6	21.7	80.3	7.0	28.0	37.3	51.6	60.3	67.4**	147.7		

* Where animals died during the course of the experiments, the number of animals still alive which are included in the average is given in parentheses.

** This value is not entirely comparable with the others since it represents females only.

TABLE II

The Gain in Weight of Individual Rats after Receiving Supplements of β -Carotene or Neo- β -carotene for 28 Days and of Negative Control Rats at Last Weighing Prior to Death

Supplement	Dose per day $\mu\text{g.}$	Gain in weight over assay period	
		Male rats	Female rats
β -Carotene	0.9	73, 68, 65, 62, 58, 55, 54, 30, Av. 58.1	67, 55, 50, 48, 47, 46, 44, 37, Av. 49.2
	2.1	93, 83, 83, 81, 80, 80, 69, Av. 81.3	83, 80, 70, 67, 65, 65, 62, 61, 57, Av. 67.1
Neo- β -carotene U*	0.9	35, 23, 21, 12, 11, -10(25), Av. 20.4	38, 35, 34, 19, 19, 18, 16, 9, 3(10), -16(20), Av. 23.5
	2.1	59, 59, 58, 55, 47, 46, 40, Av. 52.0	56, 51, 51, 50, 50, 47, 42, 40, 39, Av. 47.3
	3.3	81, 81, 75, 71, 69, 69, 67, 64, Av. 72.1	69, 55, 54, 54, 52, 49, 41, 39, Av. 51.6
	9.0		73, 72, 72, 70, 66, 62, 57, Av. 67.4
Negative controls*	0	-4(20), 0(20), -5(10), -22(20), -18(28), -16(20), -19(20)	-17(20), -9(25), -16(10), -18(28), 3(15), -26(15), -13(20), 3(10), -15(20)

* The values in parentheses are the days on which the last weight was recorded preceding death. They are not included in computing the average gain in weight for the 28-day assay period.

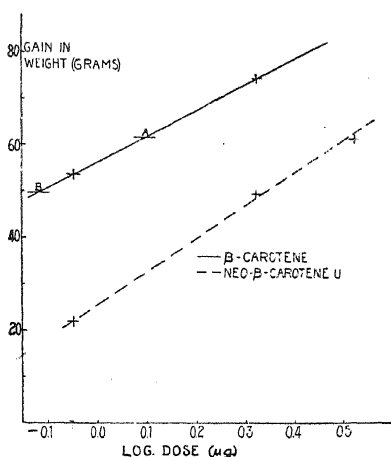


FIG. 1

3.3 $\mu\text{g.}$ of neo- β -carotene U on the β -carotene curve (point A, Fig. 1) is at a point corresponding to a log.-dose of 0.110 which is equivalent to a value of 1.29 $\mu\text{g.}$ of β -carotene. Also, the projection of the gain in weight of rats receiving 2.1 $\mu\text{g.}$ of neo- β -carotene U on the β -carotene curve (point B, Fig. 1) corresponds with a point where the log.-dose value is -0.11 (or $\bar{1}.89$) which is equivalent to 0.777 $\mu\text{g.}$ of β -carotene.

DISCUSSION

Neo- β -carotene U possesses marked potency as a provitamin A. Calculated from the gain in weight with the two intermediate dosages (where the growth levels approximate those of the β -carotene controls) the neo- β -carotene U is a little more than one-third (38%) as active as natural β -carotene.

The exact mechanism of the provitamin A activity of neo- β -carotene U in the rat is unknown. If no steric rearrangements would occur during and after the conversion into vitamin A, and if the stereoisomers of the vitamin itself could not biologically substitute for the all-*trans*-vitamin A, then one would expect the neo U to be inactive. These postulates are, however, evidently not fulfilled. An explanation for the partial loss in activity as compared with β -carotene might be a more ready destruction in the gastro-intestinal tract of the rat before absorption, or a slower absorption and greater loss by elimination. Also, it is possible that only those molecules which are re-isomerized to all-*trans*- β -carotene in the body are attacked by the enzyme and can become active.

According to Table II, in all groups (except in the tests where the rats were supplemented with 0.9 $\mu\text{g.}$ of neo- β -carotene U daily) there was a considerably greater average growth with the male rats. The average growth brought about by 1.5 I.U. and 3.5 I.U. of β -carotene are much higher than we would have supposed from earlier tests with the U.S.P. Reference Cod-Liver Oil (7). These variations are in agreement with those described by Hickman (5).

The results with Reference Oil and β -carotene are not directly comparable since in the tests with Reference Oil the supplements were administered only 24 times while in the present case 28 full doses were given. However, this variation in technic accounts for only a slight percentage of the difference noted.

SUMMARY

A crystalline stereoisomer of natural β -carotene, *viz.*, neo- β -carotene U (β -mono-*cis*- β -carotene) which contains one *cis* double bond in pri-

pheral position, has been found to have a biological activity as a pro-vitamin A which is about 38% of that of natural (all-*trans*-) β -carotene. The studies involved 103 rats.

The response of vitamin-A-deficient rats to β -carotene administered simultaneously with α -tocopherol is markedly greater than obtained in earlier tests with similar unitage of Reference Cod-Liver Oil given either with or without tocopherol. This is in agreement with the suggestion of Hickman (5) that the potency of 0.6 μ g. of β -carotene far exceeds the biological activity of one U.S.P. unit of Reference Cod-Liver Oil.

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Some Effects of Diet on the Resistance of Mice toward 2,4-Dinitrotoluene*

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INTRODUCTION AND EXPERIMENTAL

The marked effect of diet upon the toxicity and carcinogenicity of dimethylaminoazobenzene (1-9) raised the question whether the toxicity of other N-containing compounds might not be similarly affected. Dinitrotoluene (DNT) was selected for study because of its current industrial importance and because after reduction of the NO_2 groups in the body (10-14) it could theoretically yield substances related to the cleavage products of certain carcinogenic azo dyes. One route by which workmen become exposed to the compound (12), is through the skin, and dermatitis is a not-infrequent symptom of toxicity (12-15). Accordingly, the backs of 15 adult albino mice were depilated with Na_2S , and thereafter a 0.3% solution of 2,4-dinitrotoluene (Eastman tech., recryst. 3 times m.p. 71.5°) in acetone was applied with a camel's hair brush thrice weekly for two months. Later a 2.0% solution of the nitro compound was applied daily for 8 weeks. The animals remained well, and 3 raised normal litters. Other preliminary experiments revealed that adult mice remain normal when fed diets containing 0.1% DNT. Hence in subsequent experiments younger mice were employed and larger amounts of the nitro compound fed.

The diets (Table I) involved variations in the percentages of fat, protein, and carbohydrate, and also included two diets known to alter the response of rats to *p*-dimethylaminoazobenzene. The "procarcino-

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genic diet" results in the very rapid production of hepatic tumors with the azo dye (16) while the diet containing hydrogenated coconut oil represents a variation in the opposite direction (9). The experiments were performed in small series: comparable weanling mice 7.5 to 11.0 g. in weight from our stock albino colony or from the C₃H strain were fed either a diet devoid of DNT or the same diet plus 0.1% or 0.3% of the compound. All feeding was *ad libitum*, and the experiments were continued for 5 weeks. Each group contained 4 or more mice, all significant experiments were performed at least twice, and both strains of animals were used for all of the results reported.

TABLE I
Percentage Composition of Diets Employed

	Casein	Fat ¹	Yeast	Salts	Dextrin
Semi-synthetic.....	18	5 corn	8	4	65
Semi-synthetic coconut.....	18	5 coco ²	8	4	65
High protein.....	50	5 corn	8	4	33
High fat.....	18	30 Primex ³	8	4	40
Low carbohydrate.....	40	30 Primex	12	5	13
Moderate protein.....	8	5 corn	8	4	75
Low protein.....	4	5 corn	8	4	79
Procarcinogenic.....	12	5 corn	2 Vitab ⁴	4	77
High carbohydrate.....	12	—	2 Vitab ⁴	4	82
Low fat.....	18	—	8	4	70

¹ Halibut liver oil was added to the fat so that 100 g. of diet contained 3 mg. of the oil.

² A hydrogenated coconut oil obtained from Lever Bros. Co.

³ A partially hydrogenated vegetable oil.

⁴ A commercial rice bran concentrate high in all of the B vitamins except riboflavin. Diets containing "Vitab" contained no yeast.

*The growth and survival of young mice fed various diets containing
2,4-dinitrotoluene*

In the absence of the nitro compound the rates of growth were roughly similar on most of the diets: the mice usually gained over 3.0 g. per week for three weeks (Table II) while on the procarcinogenic diet, the Steenbock stock diet or the low fat diet the gains were somewhat less. When DNT was fed, however, much greater differences appeared in the growth rates of the mice on the different diets. Mice fed 0.1% DNT in diets high in fat grew almost as well as when the nitro compound was omitted from the ration, and in the presence of 0.3% DNT the mice

gained 2.0 or 2.4 g. per week. On the other hand, the growth of mice on the low fat diet was markedly diminished by the nitro compound and many died before the end of the experiment. On 0.1% of DNT 12 of 18 C₃H mice survived for 5 weeks; on 0.3% DNT only 6 of 18 C₃H mice survived; on all other diets the survival was nearly 100%.

Mice of the C₃H strain frequently grew faster than albino mice receiving the same amount of DNT, and this difference between strains was particularly evident on the procarcinogenic diet. All of 12 C₃H mice fed the diet plus 0.3% DNT survived for 5 weeks, and their average

TABLE II

Growth of C₃H Mice on Various Diets Containing 2,4-Dinitrotoluene

(Grams gain per week per mouse over a 3 week period)

Diet	No DNT g./wk.	0.1% DNT g./wk.	0.3% DNT g./wk.	Survival on 0.3% DNT*
Low carbohydrate.....	3.3	3.6	2.4	5/6
High fat.....	3.1	2.9	2.0	5/6
Steenbock stock ration.....	2.8	2.9	1.6	5/5
Commercial dog biscuit.....	3.3			
Semi-synthetic corn oil.....	3.2	2.6	1.6	6/6
Semi-synthetic hyd. coc. oil.....	3.6	2.1	1.3	3/4
High protein.....	3.3	1.8	1.2	10/10
Procarcinogenic.....	2.7	2.6	0.5	12/12
Low fat.....	2.8	(1.0)	(0.0)	6/18
Moderate protein.....	1.8	2.0	0.2	5/6
Low protein.....	0.6	0.1	0	4/4

* Numerator equals number of mice surviving during the total experimental period of 5 weeks; denominator equals total number in group. Except for the procarcinogenic diet similar results were observed in albino mice fed each of these diets.

gain per week was 0.6 g. Nine stock albino mice on this diet lost weight or failed to grow, and all died within 5 weeks. Corresponding differences were observed at other levels of DNT, although in the absence of the compound, albino mice grew as well on this diet as the C₃H mice.

Ten per cent of ethyl alcohol given *ad libitum* in place of drinking water accentuated the effects of the nitro compound. Either the alcohol alone or 0.1% of DNT alone depressed the growth rate somewhat (Table III) but the effects were slight. However, when the two agents were given together a much greater depression of the growth rate occurred. On the semi-synthetic diet or on the high fat diet the retarding effect of the two

agents together was much greater than the sum of either effect alone; on the other diets the retarding effect of alcohol plus DNT on growth was more nearly equal to the sum of the separate effects. Thus the question might be raised whether the marked sensitivity to alcohol occasionally observed in men exposed to DNT (12, 14) is due in part to the diet of the individuals concerned.

TABLE III

The Effect of Alcohol on the Growth of Mice Ingesting 2,4-Dinitrotoluene
(Grams gain per week over a 3 week period)

Diet	No DNT Water	0.1% DNT Water	No DNT 10% alc.	0.1% DNT 10% alc.	Survival* DNT + alc.
Semi-synthetic.....	2.4	2.0	2.0	-0.2	4/4
High fat.....	3.1	2.9	2.9	1.6	4/5
Low fat.....	2.7	2.0	1.4	0.9	2/6
Procarcinogenic.....	2.8	1.6	2.5	1.0	5/5

* The numerator equals the number of mice surviving for 5 weeks; the denominator equals the total number in the group.

Diet and injected dinitrotoluene

Mice grown to a weight of 20 g. or more on the various diets (Table I) were injected subcutaneously with 0.3 cc. of a solution containing 50 mg. of 2,4-dinitrotoluene per cc. of hydrogenated coconut oil. The injections were repeated on successive days for a total of three injections (total DNT = 45 mg.). Within 30 minutes after injection the animals appeared "groggy," they moved only when touched and then lapsed into semiconsciousness frequently followed by complete collapse. Breathing was shallow and rapid in some individuals, while in others it was retarded and labored. Many gasped convulsively and then remained like dead for intervals of 5 to 8 seconds. In some individuals this succession continued for hours before death supervened. In general the symptoms of toxicity lasted for 12 to 24 hours, with those animals able to survive usually showing signs of improvement by 12 hours. The urine of all injected animals was characterized by a brilliant orange pigment—a pigment incidentally also present in the urine of mice fed the larger amounts of the nitro compound.

The ability of the mice to withstand the injected DNT varied somewhat with the diet on which they had been grown (Table IV). Diets on which the mice were relatively resistant included the Steenbock stock

diet, the high fat diets and the semi-synthetic diets containing either corn oil or hydrogenated coconut oil as the source of fat. Over half of the mice fed these diets survived the 3 injections. Mice grown on the procarcinogenic diet or on the low fat diet offered the least resistance to the injected DNT, and only 2 of 22 mice on the latter diet survived the 3 injections.

TABLE IV

Effect of Diet on the Survival of 20 g. Mice Injected with 2,4-Dinitrotoluene

Diet	Survival	
	1 injection*	3 injections*
Steenbock stock.....	17/19	9/19
Semi-synthetic.....	18/19	10/19
Semi-synthetic coconut oil.....	7/8	5/8
High fat.....	15/18	10/18
High protein.....	11/19	11/19
Moderate protein.....	10/14	5/14
Procarcinogenic.....	10/19	6/19
Low fat.....	9/22	2/22

* 15 mg. DNT subcutaneously per injection. The numerator equals the number of mice that survived; the denominator, the total number in the group.

DISCUSSION

Perhaps the most general conclusion to be drawn from these experiments is that the resistance of mice to dinitrotoluene can either be increased or decreased by alterations in the diet which themselves do not effect any very great change in the growth rates of animals not exposed to the compound. The results, therefore, support the view that good growth alone is not a complete measure of the biological value of a diet.

The procarcinogenic diet appeared to have the dual property of increasing the sensitivity of rats to *p*-dimethylaminoazobenzene and of increasing the sensitivity of albino mice to dinitrotoluene. However, hydrogenated coconut oil, which increases the resistance of the rats to the azo dye (4), did not increase the resistance of mice toward the nitro compound. Thus, there appears to be no necessary parallel between the action of a dietary factor against the two toxic compounds. However, comparative studies on the pertinent B vitamins would still be of interest. The effects of diet are probably rather specific: diets high in fat are reported to increase the sensitivity of rats to trinitrotoluene (17) while in the present experiment such diets decreased the sensitivity of mice to dinitrotoluene.

SUMMARY

Mice fed 2,4-dinitrotoluene (DNT) grew better on diets high in fat than on other diets. On diets low in fat, the compound retarded the rate of growth markedly, and many mice died within 5 weeks. Mice raised to maturity on the low fat diet or on a procarcinogenic diet were less resistant to parenteral DNT than mice raised on the other diets. Mice of the C₃H strain frequently grew better than albino mice fed DNT. The difference between strains was particularly evident on the procarcinogenic diet. Ten per cent of alcohol as a drink retarded the growth rate of young mice on 4 representative diets. DNT in the diet retarded the growth rates further. The two agents together were frequently more toxic than the combined effects of each separately.

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The Inorganic Nutrient Requirements of *Escherichia coli*

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INTRODUCTION

The nutrition of *Escherichia coli* has been the subject of many investigations but no thorough attempt has been made to define its inorganic requirements. Stephenson (28) has stated that "the real salt requirements of bacteria are not known; in the absence of exact information the following are usually supplied: Na, K, Mg, Fe, SO_4 , PO_4 , and Cl. Experiments on rigorously purified salt mixtures are lacking." This deficiency in our knowledge has also been emphasized by Knight (14) and Koser and Saunders (18). The present investigation is an attempt to correct this deficiency using highly purified ingredients in a synthetic medium.

E. coli belongs to that relatively primitive group of heterotrophic bacteria which can utilize ammonia as their source of nitrogen but require an organic source of carbon. Koser and Rettger (17) used a mixture of NaCl, MgSO_4 , CaCl_2 , K_2HPO_4 , and KH_2PO_4 with glycerol and some organic nitrogenous compound. Koser (15) later omitted calcium from the medium, but left its necessity an open question. He noted that the omission of magnesium resulted in poor growth, with glucose or an organic acid as the source of carbon. The simplest medium for growth of *E. coli* contained NaCl (0.5%), $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ (0.6%), and sodium succinate (0.3%) as given by Braun and Cahn-Bronner (5). These authors suggested however that MgSO_4 (0.005%) improved growth and sometimes added "traces" of CaCl_2 and $\text{Fe}_2(\text{SO}_4)_3$. Hotchkiss (12) defined the optimum and inhibitory concentrations of a number of cations for the growth of *E. coli* on the basis of additions to a peptone broth. Under these conditions Ca acted as stimulant with optimum concentration at 0.05 M as did also Sr at 0.025 M. Other metals, such as Mn, Cu, Fe, Co, Pb, and Cd, were defined in terms of inhibitive limits. These experiments have the distinct limitation of a basal medium of indefinite composition and an inexact method of determining growth. Koser, *et al.* (16) used a partially purified synthetic medium containing NaCl, MgSO_4 , Na_2HPO_4 , and KH_2PO_4 and could find no beneficial effects on the growth of five fastidious species of bacteria from

additions of Fe, Cu, Mn, Co, Zn, Th, K, or Br. They used a range of concentration from 0.002 to 20 γ per ml. of medium.

Investigators in general have avoided the tedium and difficulties of purifying their chemicals.

EXPERIMENTAL

Strains of *E. coli* which had been isolated from fecal material, and in one case from the urine of a tuberculous patient, were obtained from the Provincial Pathological Laboratory. They were examined for contamination microscopically and by their fermentative reactions. Subcultures were made fortnightly on nutrient agar slants. Cultures in nutrient broth were carried along from day to day. Usually a fresh strain was introduced into the experiment every six months. The basal medium selected contained NaCl 0.5 per cent, $(\text{NH}_4)_2\text{SO}_4$ 0.8 per cent, KH_2PO_4 and Na_2HPO_4 0.2 per cent, and glycerol 3.0 per cent. It has been our practice to prepare this medium in ten times the above strength and to dilute and adjust to pH 6.8 electrometrically as required.

Growth Curves. Growth was followed by micro-Kjeldahl determinations (22). Special pyrex test tubes, lipped and drawn to a point at the base, were employed to permit centrifuging, washing and digesting in the same tube. A No. 24 gauge platinum wire with loop of 2 mm. diameter served for all inoculations. At least three subcultures were made in each medium before carrying out a growth curve. Points on the latter were determined by removing two tubes at intervals from the incubator kept at 37°C. They were heated in a water bath at 60°C. for 20 minutes and placed in a refrigerator at 4°C. until analyzed.

Reagents and Apparatus

All glass apparatus was first soaked in acid dichromate cleaning mixture, rinsed thoroughly with water, washed with soap and water, rinsed with tap water, distilled water and finally redistilled water. The method of Richards (24) of centrifugal drainage was generally applied for recrystallizations. Laboratory distilled water was twice redistilled in an apparatus described by Coombs (7) as for conductivity water (13). Over a year was required in preparing the chemicals of the basal medium. A state of purity comparable with that for atomic weight determinations was sought. Only the general procedures followed are given below.

Sodium chloride. The method of preparation was essentially that of Richards and Wells (26) from metallic sodium and HCl gas with two fusions in platinum vessels and five crystallizations.

Ammonium Sulfate. Kahlbaum's "zur Analyse" grade was purified with KMnO_4 after the practice of Richards, Kothner, and Tiede (25). The recrystallized NH_4HSO_4 was converted to $(\text{NH}_4)_2\text{SO}_4$ with gaseous NH_3 and the latter precipitated in excess of NH_3 (24%).

Sodium and potassium phosphates. Merck's "reagent" chemicals, after Sørensen, were purified by the method Baxter and Jones (2) treating with purified H_2S and recrystallizing six times.

Glycerol. Merck's analytical grade was steam distilled in an all-glass apparatus constructed after that of Langmuir (19). Filtered super-heated steam at 255°C. was passed through the glycerol in a Claissen flask in an oil bath at 185°C. The

vapor was carried into a double condensing system consisting of an air-cooled tower and a worm water-cooled condenser the end of which was inserted in a balloon flask connected to an aspirator pump. The temperature of the steam was kept at 180°C. and the pressure in the apparatus at 60 mm. of mercury. The product obtained from the air condenser was concentrated *in vacuo* with the aid of a fine stream of nitrogen at a pressure of 40 mm. to a specific gravity of 1.25. The product was fractionally distilled at 1 mm. pressure in a stream of purified, dry nitrogen in an all-glass apparatus of our own design. The middle fraction was collected and redistilled. The final product had a specific gravity of 1.261, which, according to the tables of Bosart and Snoddy (4), represents a concentration of 99 per cent. The tests listed in Table I were all negative and served to fix the upper limits of possible contamination.

Sterilization. Medium Berkefeld candles were used initially after very careful cleaning but consistent results could not be obtained with different filters. It was found that new ones did not give results consistent with the old ones. *When only traces of salts are important it would seem impossible to obtain a chemically clean candle.* A Seitz filter was also tried but this was even less satisfactory. On auto-

TABLE I
Concentration of Impurities in the Basal Medium by Qualitative Tests

Metal	Test	Sensitivity	Upper Limit in Basal Medium γ/ml.
Calcium.....	Grégoire, <i>et al.</i> (9)	6 in 10 ⁷	<0.06
Magnesium.....	Barnes (1)	1 in 10 ⁶	<1
Copper.....	Thomas and Carpentier (30)	1 in 10 ⁷	<0.01
Iron.....	Lyons (21)	1 in 10 ⁶	<1

claving the concentrated basal medium a fine white precipitate formed. Finally the following procedure was adopted. All flasks to be used were filled with cleaning fluid and autoclaved at 20 lbs. pressure for 30 minutes. They were washed thoroughly, filled with distilled water and reautoclaved. This was repeated with fresh distilled water. The concentrated basal medium was prepared in sterile triple-distilled water and kept in a refrigerator in sterile glassware without further sterilization. It was found to remain sterile over a period of six weeks, and from it the dilute medium could be made and autoclaved.

Growth on the Purified Medium

The growth curve on a medium prepared with the specially purified chemicals (*X*) was first compared with one containing "analytical" chemicals (*R*). These in turn were contrasted with the growth curve on Difco nutrient broth (*N.B.*) and a nutrient broth medium made to include NaCl at 0.5 per cent and the phosphate buffer salts at 0.2 per cent in comparison with medium *R*. An inoculum from nutrient agar was

carried through 36 subcultures on medium *R* before determining the growth curve. Medium *X* was inoculated from medium *R* and a growth curve determined after 6 subcultures. The results are shown in Figs. 1 and 2.

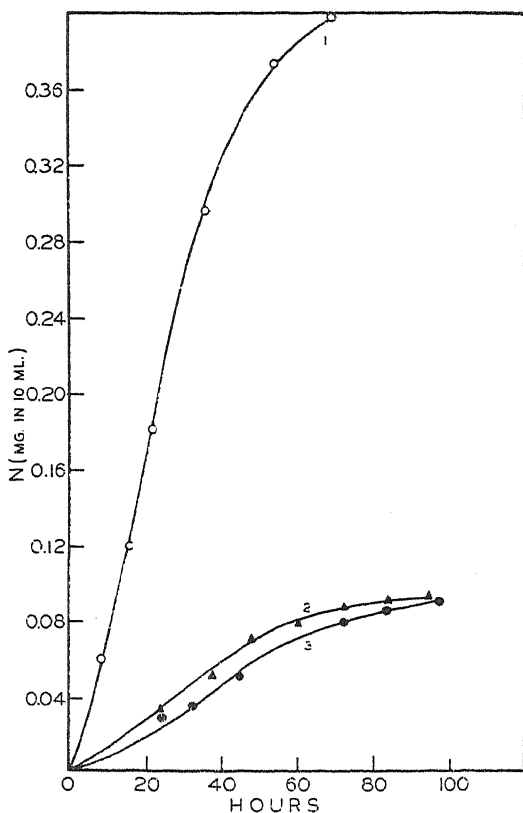


FIG. 1

Growth Curves of *Escherichia coli*

on (1) Nutrient Broth

(2) Basal synthetic medium (*X*) of highly purified chemicals,

(3) Basal synthetic medium (*R*) of "analytical" chemicals.

It is apparent that both synthetic media permit growth but at a rate much reduced as compared with nutrient broth. They are definitely deficient. There exists apparently no difference between the two syn-

thetic media. This is surprising in the light of the probable difference in concentrations of impurities. Either the concentrations of traces of impurities in medium *R* were so low as to be beyond the effective range for *E. coli* or some one limiting factor was absent in both media.

The synthetic medium *R* now provided a systematic approach to the problem of modifying it to equal medium *N.B.* An initial exploratory experiment was carried out by permitting growth for 48 hours in medium *R* to which were added various inorganic constituents at their probable effective concentration. These were Fe 0.05, Ca 1.0, Mg 0.2, Mn 0.01, Zn 0.01, Cu 0.005 as mg. in 10 ml. The results in terms of total nitrogen are expressed in Table II and they suggest immediately the importance of magnesium in the metabolism of *E. coli*.

TABLE II

Growth of E. coli after 48 Hours on the Synthetic Medium (R) with Additional Metallic Salts

Medium	Nitrogen mg./10 ml.
Control.....	0.072
Control + Fe.....	0.075
Control + Mg.....	0.260
Control + Fe, Ca.....	0.065
Control + Fe, Ca, Mg.....	0.260
Control + Fe, Ca, Mg, Cu.....	0.245
Control + Fe, Ca, Mg, Cu, Mn.....	0.245
Control + Fe, Ca, Mg, Cu, Mn, Zn.....	0.260
Nutrient Broth.....	0.357

Growth on Basal Medium (R) Supplemented by Single Metallic Ions

A systematic investigation was now undertaken to determine the effect on the growth curve of adding varying concentrations of single purified salts to the basal medium *R*, by the procedure described above.

We have at times shortened this procedure by inoculating six culture tubes from the third subculture and determining the nitrogen content in duplicate tubes at three different times, usually 45, 90, and 120 hours. This yielded three points on the growth curve and was a satisfactory, initial guide.

Magnesium. Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck reagent), was recrystallized twice from distilled water, washed centrifugally and dried. This salt was made up in the concentrations shown in Table III. Growth was obtained in all of these media and a complete growth curve

was done at 5 γ per 10 ml. as shown in Fig. 2. This shows a long "lag" phase of about 60 hours followed by the logarithmic phase to 135 hours developing into the characteristic irregularities of late cultural growth.

TABLE III

Effect of Different Concentrations of Magnesium on Growth at 135 Hours

<i>g./10 ml.</i>	<i>γ/10 ml.</i>	Total N <i>mg./10 ml.</i>
2×10^{-3}	200	0.124
5×10^{-5}	5	0.117
5×10^{-6}	0.5	0.097
5×10^{-7}	0.05	0.083
5×10^{-8}	0.005	0.070
5×10^{-9}	0.000,5	0.069
5×10^{-10}	0.000,05	0.058
5×10^{-11}	0.000,005	0.045
5×10^{-13}	0.000,000,05	0.040
Control.....		0.035

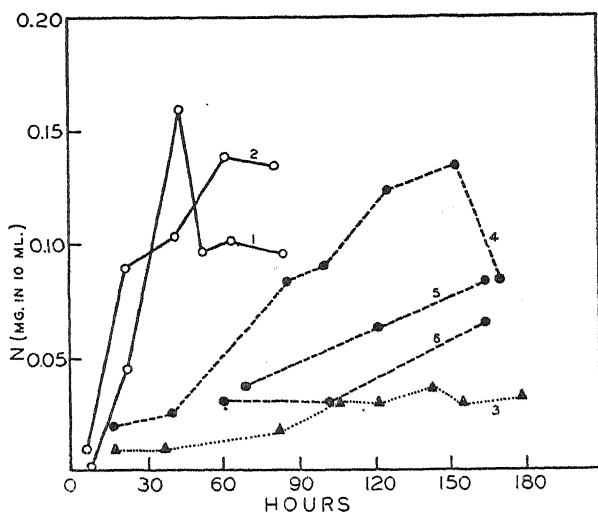


Fig. 2

Growth Curves

on (1) Nutrient Broth

(2) Nutrient Broth with NaCl added at 0.5% and phosphates at 0.2%

(3) Basal synthetic medium (*R*)

(4) Medium *R* with Mg at 5 γ per 10 ml.

(5) Medium *R* with Mg at 0.000, 5 γ per 10 ml.

(6) Medium *R* with Mg at 0.000,05 γ per 10 ml.

At 500 γ no acceleration in any phase was apparent. Below 5 γ the slope of the logarithmic phase was less steep, until at 0.000,005 γ and below, it corresponded with the basal medium.

Calcium. Calcium chloride, CaCl_2 (B.D.H. analar), was recrystallized twice from distilled water, washed centrifugally and dried carefully at 500°C. This salt was added to the basal medium in concentrations of 175, 16, 1.6, 0.16 γ per 10 ml. Growth was obtained in all of these tubes. A complete growth curve was done at a concentration of 1.6 γ as shown in Figure 3. At no concentration was there any significant difference in growth from that on the basal medium.

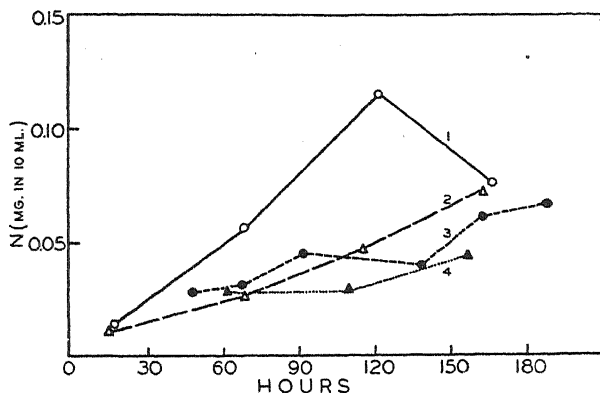


FIG. 3
Growth Curves on Basal Medium R

supplemented with

- (1) Silicon, at 1848 γ
- (2) Iron, at 14 γ
- (3) Calcium, at 16 γ
- (4) Calcium, at 1.6 γ

Rubidium and Caesium. Rubidium chloride (Schuchardt) without purification was added to the basal medium in concentrations of 144 and 1.4 γ per 10 ml. Two curves of three points each were determined. They were very similar and tended to be slightly higher than those of the basal medium alone. The difference was so slight however as to be attributable to the presence of slight impurities, such as magnesium.

Caesium chloride (Schuchardt) was also added to the basal medium at levels of 160 and 1.6 γ per 10 ml. Three-point curves showed no difference from the controls.

Strontium. Strontium chloride, $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ (Merck reagent), was recrystallized twice from distilled water, washed centrifugally and dried. Added to the basal medium at levels of 769 and 0.769 γ per 10 ml. it showed no significant difference from the control.

Iron. Ferrous ammonium sulfate, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (Merck reagent), was recrystallized three times from distilled water, acidified with a few drops of sulfuric acid. It was added to the basal medium in concentrations of 14, 3.5, 0.005, 0.0005 γ per 10 ml. The most effective concentration was 14 γ and a complete growth curve with 3.5 γ showed a slight increase over the control as in Fig. 3.

Cobalt and Nickel. Cobaltous sulfate, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck reagent), was recrystallized four times. It was added to the basal medium in concentrations of 223, 133, 67, 22, 2.2, 0.022 γ per 10 ml. All growth was inhibited at 22 γ or more. Growth proceeded at the rate of the control at 2.2 γ and 0.022 γ . These observations were repeated with the same result showing a rather sharp inhibitive concentration at 22 γ .

Nickel sulfate, $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ (Merck reagent), was recrystallized four times. The purified salt was then added to the basal medium in concentrations of 41.6, 4.1, 0.041, 0.0041 γ per 10 ml. At the two higher concentrations growth ceased in the second subculture. At the two lower the curve showed no significant difference from that of the basal medium. Nickel was thus comparable with cobalt.

Copper. Copper sulfate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Merck reagent), was recrystallized three times. It was added to the basal medium in concentrations of 125, 12.5, 0.7, 0.12, 0.0012, 0.000,012 γ per 10 ml. These concentrations had no apparent effect on the growth rate.

Silicon. Sodium silicate, $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ (Baker c.p.), was recrystallized three times from dilute sodium hydroxide with some difficulty, washed carefully, and added to the basal medium at concentrations of 1,840 and 1.84 γ per 10 ml. In both cases additional growth was obtained but because of the difficulty inherent in purification of the silicate it was not taken to be significant (see Fig. 3).

Zinc. Zinc sulfate, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck reagent), was recrystallized three times. This was used in concentrations of 56 and 0.056 γ per 10 ml. There was no significant effect.

Aluminum. Aluminum sulfate, $\text{Al}_2(\text{SO}_4)_3$ (Baker c.p.), was recrystallized twice. In concentrations of 19.3 and 0.19 γ per 10 ml. there was no significant stimulation of the growth rate.

Manganese. Manganous sulfate, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (Merck reagent), was recrystallized three times and used in the concentrations of 500, 50, 5, and 0.05 γ per 10 ml. The highest concentration produced a considerable precipitate of manganous phosphate and could not be used. The others were almost identical, with slight, if any, improvement over the basal rate.

Molybdenum. Molybdic acid, $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$ (Merck reagent), was recrystallized once from ammonium hydroxide, washed centrifugally and dried. It was then added in concentrations of 66.6, 0.66, and 0.0066 γ per 10 ml. to the basal medium. Three-point curves showed no significant increase in growth.

A comparison of the concentrations used is presented in Table IV.

Growth on Basal Medium (R) Supplemented by Two Metallic Ions

Since magnesium ions had such a marked effect on the rate of growth, it was thought that this ion might easily be a limiting factor in testing the effect of other ions. It was therefore necessary to test the influence of the more probable metallic ions supplementing a medium containing magnesium.

Magnesium and Iron. The basal medium containing magnesium at 5 γ per 10 ml. was prepared with the addition of iron at 14, 5.6, 0.5, 0.005, and 0.000,5 γ per 10 ml. The results are shown graphically in Fig. 4. The higher concentrations induced a marked increase in the rate of growth. These growth curves were comparable with that on

TABLE IV
Concentration of Ions Tested Without Stimulating Effect

		Concentration $\gamma/10$ ml.	
Ion	Calcium.....	175	—0.16
	Strontium.....	769	—0.769
	Rubidium.....	144	—1.4
	Caesium.....	160	—1.6
	Copper.....	125	—0.000,012
	Silicon.....	1,840	—1.84
	Zinc.....	56	—0.056
	Aluminum.....	19.3	—0.19
	Manganese.....	500	—0.05
	Molybdenum.....	66.6	—0.006,6
	Cobalt.....	223	—0.022
	Nickel.....	41.6	—0.004,1

nutrient broth with perhaps the peak attained about 20 hours later. The latter could be placed about 45 hours in the nutrient broth and about 65 hours in the synthetic medium. The effect of iron was still apparent at a concentration of 0.005 γ but below this the growth curve was equivalent to the effect of the magnesium alone as shown in Fig. 2, except that the logarithmic phase came earlier in the presence of iron. Higher concentrations of iron could not be tried because of the precipitation of iron phosphate. Even at 14 and 5.6 γ a slight opalescence developed in the media.

Another series of curves was obtained keeping the iron concentration constant at 1.4 γ per 10 ml. and varying the magnesium between 5.0 and 0.000,5 γ per 10 ml. Three-point curves showed gradually decreasing growth rates with the greatest effect between 5 and 0.5 γ .

Magnesium and Other Metallic Ions. Concentrations of magnesium with other metals were tried with magnesium at 5 γ and zinc at 5.4 and

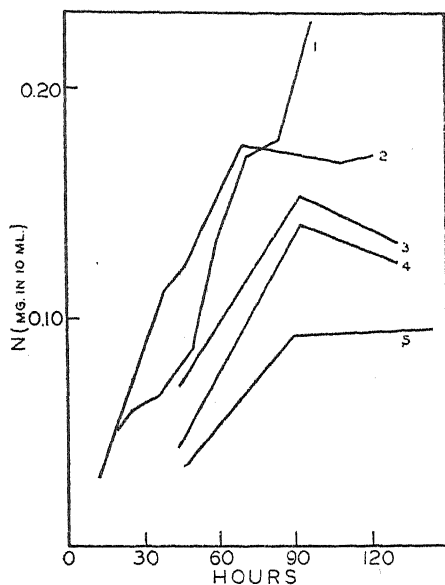


FIG. 4

Growth Curves on Basal Medium (R) supplemented with Magnesium at 5 γ per 10 ml. and Iron at

- (1) 14 γ
- (2) 5.6 γ
- (3) 0.5 γ
- (4) 0.005 γ
- (5) 0.0005 γ per 10 ml.

TABLE V

Effect of Combinations of Metallic Ions

Ions	Depressing Range $\gamma/10$ ml.	No Effect $\gamma/10$ ml.
Mg—Zn	Mg 5; Zn 5.4	Mg 5; Zn 0.056
Mn—Fe		Mn 5; Fe 0.5
Mg—Co	Mg 5; Co 22	Mg 5; Co 2.2
Mg—Ni	Mg 5; Ni 0.04–41.6	
Mg—Mn	Mg 5; Mn 50	

0.056 γ ; manganese at 50 and 5 γ ; cobalt at 67 and 2.2 γ ; nickel at 41.6 and 0.04 γ ; with the results as shown in Table V.

An experiment was tried to determine whether manganese could replace magnesium in the presence of iron with manganese at 5 γ per 10 ml. and iron at 0.5 γ . Two growth curves on this medium were determined but they were no better than with iron alone.

Manganese was also studied as a substitute for iron in the presence of magnesium. Five growth curves were run with contradictory results. Two were similar to the effect of magnesium alone. In three experiments stimulation was definite although the lag phase was prolonged. This medium has given the most inconsistent results and it is not possible to conclude from our evidence that manganese is a substitute for iron.

Growth on Medium X Supplemented by Magnesium and Iron

The stimulating action of both magnesium and iron has been clearly established when added to the basal medium (*R*) made from chemicals of "analytical" grade. Similar growth curves had previously been obtained with the basal medium (*R*), unsupplemented, and medium *X* made from highly purified chemicals. It was important to show that similar curves comparable with those on nutrient broth (*N.B.*) could be obtained if both synthetic media were supplemented with magnesium and iron. This was therefore done with magnesium at a concentration of 5 γ and iron at 5.6 γ per 10 ml. The results are shown in Fig. 5. Growth on medium *X* was as good as on medium *R* while both were comparable with medium *N.B.* although not showing quite as steep a curve in the logarithmic phase. This may indicate the time required for the synthetic mechanism to function in producing necessary amino acid "activators" (27).

As the specially purified chemicals were considerably purer than the "analytical" chemicals this experiment may be taken to indicate that no essential unknown nutritional element was present in medium *R*. This conclusion would have to be modified if the amount required were of an exceptionally low order of magnitude.

Initial Inoculation. Throughout this investigation the initial inoculum for all growth curves has been a 2 mm. loopful of the medium under trial in subculture. As the rapidity of growth has varied enormously, the number of cells in any inoculum must have varied likewise. An experiment has been carried out in an endeavor to equate the milligrams of nitrogen in a loopful to the number of viable cells. Medium *R*, supplemented with 5 γ of magnesium per 10 ml. and varying amounts of iron, was tested. Four tubes were removed from the incubator at inter-

vals; plate counts and micro-Kjeldahl determinations were done in duplicate. Results of a typical experiment, shown as averages, are

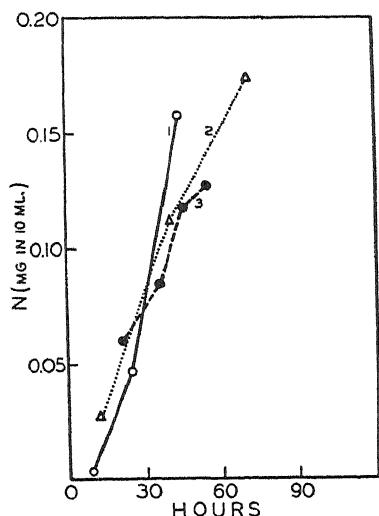


FIG. 5

Growth Curves

- on (1) Nutrient Broth
 (2) Basal Medium *R* supplemented with Mg at 5 γ and Fe at 5.6 γ per 10 ml.
 (3) Basal Medium *X* also supplemented with Mg and Fe.

TABLE VI

Relation of Counts of Initial Inoculum to Rate of Growth

Iron $\gamma/10$ ml.	Counts of Initial Inoculum	Counts at 35 Hours
0.005	500,000	240×10^6
0.5	750,000	$18,500 \times 10^6$
14.0	470,000	$20,500 \times 10^6$

TABLE VII

Comparison of Cell Counts and Total Nitrogen

I			II		
Time hrs.	Counts $\times 10^6$	Total Nitrogen mg.	Time hrs.	Counts $\times 10^6$	Total Nitrogen mg.
12	240	0.0239	10	45	0.0057
22	60	0.0455	21	750	0.0201
34	8,400	0.0682	34	1,100	0.0438
46	410	0.0782	—	—	—
70	440	0.0988	79	23	0.0955

listed in Table VI. The *number* of cells in the initial inoculum is apparently not important relative to the *medium* for growth.

Relation of Viable Cells to Total Nitrogen

A further series of experiments was done in an endeavour to determine, over the period of incubation for growth curves, the relation of the micro-Kjeldahl values to the number of viable cells. The basal medium *R* with magnesium at 5 γ and iron at 5.6 γ per 10 ml. was chosen. The results of two experiments are shown in Table VII. In both series the largest number of viable cells was present at about 34 hours. After this the cellular nitrogen continues to increase but a high rate of death ensues. The accumulation of dead cells must therefore contribute to the values recorded in many experiments in this investigation to a large extent. Such a criterion is nevertheless an index of the power of promoting growth in the medium employed. It is a measure of the *total protoplasmic mass* as against the *number* of cells capable of division under new conditions.

Adaptation to Synthetic Medium

As many of the experiments recorded above have been done on deficient media, as is the case for the basal medium, it was essential to determine whether any adaptation took place in the strain of *E. coli* used. The organism was passed through 21 subcultures on the basal medium and then inoculated in this medium supplemented with magnesium (5 γ) and iron (5.6 γ). The growth curves were the same as those obtained on direct inoculation of this medium. Thus the total nitrogen at 48 hours was 0.121 mg. and this corresponds with the value shown in Fig. 4.

DISCUSSION

The rate of growth of our strains of *Escherichia coli* on the basal medium was relatively very slow. The organism however did have the ability to exist on this medium and thus to provide a sensitive response to traces of salts which had metabolic significance. Our findings of magnesium and iron *only* as supplementary elements necessary for optimum growth fit remarkably well with recent observations of Werkman and his collaborators.

The products of decomposition of glycerol were shown by Harden (10) to be essentially the same as from glucose. This fermentation takes place with difficulty anaerobically (29) and appears to require a hydrogen acceptor for the oxidation of glycerol. This could be supplied by the oxygen of the air if the proper enzymic system were present.

E. coli has been shown to possess a primitive cytochrome system (b and d) (8). Cells of *Aerobacter* grown in the absence of iron, do not possess any detectable spectral absorption at 560 or 590 $m\mu$ and are deficient in catalase (34). This organism was found to require 0.025 p.p.m. of iron for optimum growth. Waring and Werkman (35) have also estimated the iron requirement of *E. coli* at 0.02 to 0.03 p.p.m. and found a slight depressant effect at 5 p.p.m. Expressed in this way in conjunction with magnesium at 0.5 p.p.m. our results showed optimum growth at 0.56 to 1.4 p.p.m. as shown in Fig. 4 and a slight effect at 0.0005 p.p.m. Lack of iron under these conditions would thus be equivalent to anaerobic conditions.

The chain of reactions in the degradation of glycerol beyond the formation of glycerose may be conceived as consisting of phosphorylation and conversion to phosphoglyceric acid, to phosphopyruvic acid, to lactic acid, acetic acid, ethanol, and carbon dioxide. Evidence for the presence of phosphatase in the cells of *E. coli* has been adduced by Boivin and Mesrobian (3). Bacterial phosphatases appear to be greatly accelerated in their function by the presence of Mg^{++} (23). This conception also fits into the work of Leinbrock (20) on the intermediary metabolism of carbohydrates of the colon bacteria as being comparable with degradation in muscle. Finally the intermediate steps have been studied by Utter and Werkman (31, 32) with cell-free extracts of *E. coli* and specific substrates. They have shown that Mg^{++} and Mn^{++} in concentrations of 0.005 *M* facilitate the rapid establishment of the equilibrium of 2-phosphoglyceric acid and enol-phosphopyruvic acid by enolase. They have also found that Ca^{++} and Ni^{++} inhibit this reaction. Our experiments showed Mg^{++} most effective for growth about 0.5 γ per ml. which is 0.000,02 *M*. This may be taken as a reasonably good agreement for the measurement of the effect under very different experimental conditions. It is also of interest that the enzyme enolase has been crystallized by Warburg and Christian (33) and found to be a metallo-protein complex of Mg. As regards the efficacy of Mn^{++} , despite repeated trial, our results were never sufficiently conclusive to permit a confirmation on the basis of growth curves. There is thus ample physiological evidence to confirm and explain our findings that both magnesium and iron are elements necessary in traces to support optimum growth of *E. coli* in addition to Na, K, Cl, SO_4 , and PO_4 . The order of magnitude of these requirements for iron and magnesium of *E. coli*

at 5.6 and 5 γ per 10 ml. is in agreement with that found by Horner and Burk (11) for *Azotobacter* at 7.7 and 50 γ per 10 ml.

Iron has been found to function in the growth of *E. coli* at concentrations between 0.0005 and 14 γ as seen in Fig. 4. Taking the middle range of 0.005 and 0.5 γ of iron per 10 ml., assuming assimilation of all available iron and equal distribution among the viable cells, the lower concentration represents 220,000 and the higher 250,000 haematin molecules per cell for counts at 35 hrs. These figures are comparable with the figure of 10,000 molecules of molybdenum calculated by Burk (6) for maximum nitrogen fixation in a cell of *Azotobacter*.

We wish to acknowledge our indebtedness and to express our gratitude to the Associate Committee on Medical Research of the National Research Council of Canada for financial grants received by one of us (E. I. P.) during the course of the work.

SUMMARY

The inorganic requirements for normal growth of *Escherichia coli* have been determined on a synthetic medium containing NaCl, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , Na_2HPO_4 , and glycerol by adding inorganic supplements. All constituents of the basal medium were highly purified. Growth curves were constructed from micro-Kjeldahl estimations. Similar curves showing deficient growth were obtained on the basal medium made from the highly purified chemicals and "analytical" chemicals as compared with growth on nutrient broth.

Magnesium and iron have been shown to be necessary elements for normal growth. The optimum concentration of magnesium was about 5 γ per 10 ml. of medium but a response was detectable as low as 0.000,05 γ . The optimum concentration of iron was about 5 to 14 γ with a response detectable at 0.005 γ . Growth on the basal medium supplemented by magnesium and iron was comparable with that on nutrient broth except that the lag phase was prolonged by 20 hours and the peak came 65 hours after inoculation.

Calcium, strontium, cobalt, nickel, manganese, zinc, aluminum, copper, rubidium, caesium, silicon, and molybdenum gave no stimulation to growth when added alone over a wide range of concentrations. This was also true for manganese, zinc, cobalt, and nickel when added together with magnesium.

An explanation of these findings is proposed on the basis of the re-

quirement of iron in the cytochrome system necessary to initiate the dehydrogenation of glycerol, and the requirement of magnesium in enzymic mechanisms of phosphorylation and degradation of glyceric acid.

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Tissue Lipids in Ascorbic-Acid-Deficient Guinea Pigs¹

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Terbruggen (1) noted that livers of scorbutic guinea pigs contained 10 to 19% of fat in contrast to a normal fat content of 3.4 to 3.8%. Fatty infiltration of the liver in guinea pigs having varying degrees of scurvy was observed and described by Bessey, Menton and King (2) in 1934. Spellberg and Keeton (3) found, in 1939, that the livers of guinea pigs deprived of vitamin C or receiving subminimal amounts of pure ascorbic acid and dying from scurvy contained very high percentages of fat as compared to the livers of animals receiving sufficient ascorbic acid. The effect of inanition was studied in animals fed only ascorbic acid and water. Under these conditions, large amounts of fat were not observed in the livers.

A paired feeding experiment was conducted by MacLean, Sheppard and McHenry (4) to determine the effect of scurvy and inanition on guinea pig tissues. No chemical analyses were made for the fat content of the livers or adrenal glands, but upon microscopic examination, nearly all the animals in the scorbutic group appeared to have normal livers.

Longenecker and Weissert (5) observed the development of intensely fatty livers in scorbutic guinea pigs fed the Sherman assay diet (6) alone or supplemented with subminimal amounts of ascorbic acid. Spellberg and Keeton (7) again reported in 1940 that fatty and fibrotic livers had been produced in guinea pigs and rabbits on a scor-

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butogenic diet, whereas starving animals or those on a normal diet showed no such effects. However, the latter authors showed that some factor other than vitamin C-deficiency must have caused the fatty livers because added ascorbic acid, orange juice, lipocaine, choline, yeast, and dextrose were without appreciable effect in preventing fatty livers in guinea pigs on a modified Sherman diet.

In 1941, Baldwin (8) conducted a paired feeding experiment similar to that of MacLean, Sheppard and McHenry. The Sherman diet was fed to two groups of guinea pigs which had been paired according to gain in body weight during a preliminary period, final body weight, and sex. Both groups consumed the same amounts of the basal ration. Each animal in one group received daily two milligrams of ascorbic acid; the second group received no vitamin C. Food consumption of the first group was restricted to that of the paired animal in the second group. The average amount of total lipids in the livers of those animals receiving no vitamin C was $4.6 \pm 1.4\%$; for the group receiving vitamin C, the total liver lipid was $5.7 \pm 2.2\%$. Furthermore, no intensely fatty livers such as those observed in previous investigations were found in any of the animals. The average adrenal lipids for the scorbutic group and the control group were, respectively, 20.3 and 22.5% of the weight of the adrenal tissue.

In a later paper, Spellberg, Keeton, and Ginsberg (9) suggested that the liver may have difficulty in metabolizing certain types of fat. When these fats become fixed in the hepatic parenchyma, permanent damage results.

Since the completion of our studies, Beyer (10) attempted to clarify the uncertainty about the effect of avitaminosis C and obtained results similar to those shown below. His data show that the hepatic lipid contents of normal guinea pigs, those receiving injections of vitamin C while being fed the scorbutogenic diet, and those on the basal diet without supplemental vitamin C were essentially the same.

Inanition and fasting have been shown many times to cause fatty infiltration of the liver. Inasmuch as inanition always accompanies acute scurvy, it is difficult to ascertain the cause of increased fat in the livers of scorbutic animals unless steps have been taken to rule out the factor of inanition. Only one reported study (4) has been carried out in such a manner but no chemical analyses of the hepatic lipid were made. Microscopically, however, no significant differences were evident between the livers of scorbutic and control guinea pigs.

The amount of adrenal cholesterol has also been variously reported to increase or decrease during scurvy and fasting. Therefore, this investigation was undertaken to study the liver, adrenal, and carcass lipids in vitamin C-deficient guinea pigs in which the effect of inanition accompanying the onset of scurvy was eliminated by the use of the paired feeding technique.

EXPERIMENTAL

Animal Technique

Preparation of Animals. Young guinea pigs with an average weight of 245 grams were housed in individual galvanized iron cages with raised screen floors and were fed *ad libitum* a basal diet consisting of the following ingredients:

Rolled oats	3000 g.
Bran	3000 g.
Skim milk powder (heated for 8 hrs. at 100°C.)	3000 g.
Butterfat	1000 g.
Irradiated Brewers' yeast	400 g.
Sodium chloride	100 g.

For twelve days previous to pairing, the animals received this diet supplemented with fresh spinach and ascorbic acid. Crystalline vitamin C³ was dissolved in distilled water daily and aliquots containing five mg. were fed by pipette to each guinea pig. This procedure was designed approximately to saturate the tissues with vitamin C so that all the animals would be on a comparable basis at the beginning of the experimental period. A record of growth during this pre-experimental period was kept for use during the subsequent pairing of the animals. Animals whose growth performances were not satisfactory were discarded.

The animals were divided into two groups which were matched not only for sex and weight but also for gain in weight during the pre-experimental twelve-day period. The average weights of the two groups were identical, but Group A (the deficient group) showed a slightly greater gain in weight than Group B (control group).

Paired Feeding Technique. All the animals were continued on the basal diet. Each animal in Group A received a weighed portion of food which was eaten *ad libitum*. The following day the food which had not been consumed was weighed, and the animals in Group B were then fed the same amount of food as was eaten the previous day by the corresponding animals in Group A. (Thus, each animal in Group A consumed as much as its mate in Group B consumed or more than this amount.) The twenty-five animals in Group A received no supplement to the basal ration, whereas a freshly prepared solution containing 2 mg. of ascorbic acid was fed by pipette daily to each of the animals in Group B.

³ Donated by Charles Pfizer.

Growth curves were plotted for each animal; those for the animals of Group A were very similar to the growth curves described by Sherman and Smith (11) for scorbutic guinea pigs. Steady growth was observed for the deficient animals to the twelfth and thirteenth days when a rapid weight loss started. Similar growth was noted for the corresponding animals in Group B, but the decline did not occur until two or three days later when food consumption for both groups was substantially decreased.

Development of Scurvy and Autopsy of Animals. The deficient animals showed outward signs of scurvy at the end of approximately fifteen days, and frank scurvy was evident a few days later. No symptoms of scurvy developed in any of the animals in the control group. When the guinea pigs in Group A were in the last stages of scurvy, they and their mates from Group B were anaesthetized with chloroform and sacrificed by decapitation.

Autopsies were performed to determine the extent of scurvy development; the livers and adrenals were removed, freed from connective tissue and weighed for the immediate extraction of lipids (Table I). Two livers from animals (A-13 and A-21) showed cirrhosis and the entire liver of animal A-13 appeared granular.

The extent of scurvy was determined by a system of "scoring" similar to that used by Kenny (12) and by Sherman and Smith (11). The average scurvy score for the animals of Group A was 15 to 16 out of a maximum possible score of 24; that of Group B was less than one.

Extraction of Lipid Material from Tissues

Total Liver Lipid. The livers were minced separately with acetone in a Waring Blendor and transferred quantitatively to 300 ml. fat-extraction flasks. The individual ground livers were refluxed with three successive portions of acetone for 30 minutes followed by three successive portions of petroleum ether. The total acetone extract was concentrated nearly to dryness, and the residue was added to the total ether extract. The water-soluble impurities in the ether extract were removed by washing with distilled water. After removal of the petroleum ether by distillation *in vacuo* on a steam bath, total lipid was obtained by direct weighing.

The average total lipid per 100 g. of liver tissue was somewhat higher for the animals of Group B (4.96 ± 2.16 g.) than for the deficient animals (3.71 ± 1.05 g.), but the difference was not significant. The average weights of livers in Group A (11.41 g.) was higher than for Group B (9.18), and the liver lipid per 100 g. of animal was considerably higher for Group A than for Group B (178 mg. and 141 mg., respectively). See Table I.

Total Adrenal Lipid. The adrenal glands were ground separately in a mortar, and the same procedure was used for the extractions as was

TABLE I

Animal and Tissue Weights and Tissue Lipid Content

Animal pair	Weight at death		Weight of livers		Liver lipid		Weight of adrenals		Adrenal lipid	
	A	B	A	B	A	B	A	B	A	B
	g.	g.	g.	g.	per cent	per cent	g.	g.	per cent	per cent
1	278	358	12.21	11.06	5.22	8.58	0.251	0.211	24.1	21.1
2	264	262	13.01	8.52	7.09	2.77	.266	.173	19.3	21.7
3	324	337	16.82	11.56	3.86	4.40	.246	.226	19.2	23.8
4	276	370	14.09	10.83	3.59	5.18	.234	.169	21.5	24.6
5	210	*	9.94		4.53		.243		22.8	
6	258	305	11.70	9.63	4.93	3.45	.309	.217	28.9	18.9
7	306	353	13.61	10.46	3.39	4.24	.276	.261	19.6	21.3
8	241	325	11.80	10.42	3.96	7.02	.228	.227	19.8	26.0
9	225	335	10.57	10.94	3.54	6.96	.344	.182	14.8	26.0
10	275	273	11.68	9.27	3.68	1.81	.251	.242	17.0	22.2
11	209	309	11.38	10.59	3.06	5.41	.234	.185	14.6	27.8
12	190	254	9.54	7.80	4.12	3.01	.188	.166	16.6	20.3
13	245	340	13.30	12.60	2.02	3.85	.246	.160	17.3	21.2
14	205	273	9.22	12.51	3.52	4.14	.172	.173	26.6	22.5
15	231	252	10.64	8.98	3.55	3.05	.276	.147	20.6	16.5
16	208	261	10.07	6.20	3.67	3.45	.177	.134	21.1	29.3
17	207	254	9.82	8.70	4.45	11.10	.316	.205	17.7	24.2
18	203	267	11.43	8.40	3.34	4.88	.191	.176	20.9	25.8
19	170	235	6.39	8.83	3.04	2.75	.264	.184	16.2	23.9
20	214	*	11.09		4.48		.233		29.6	
21	257	341	14.77	10.31	3.61	5.48	.338	.178	25.0	26.6
22	180	355	6.82	10.00	2.47	5.33	.255	.161	20.6	21.6
23	183	276	8.90	†	2.89		.392	.223	19.4	29.3
24	200	*	8.60		3.39		.235		23.5	
25	192	262	8.30	8.78	2.79	0.60	.246	.185	17.1	26.1
Ave.			11.41	9.82	3.71	4.96	.257	.182	20.5	23.7
					±1.05	±2.16			±0.8	±0.7

* Animal died before completion of the experiment.

† Poisoned accidentally with chloroform.

used for the extraction of the liver lipids. The average lipid per 100 g. of adrenal tissue was also higher for Group B (23.7 ± 0.7 g.) than for Group A (20.5 ± 0.8 g.) but not significantly so. The adrenals of Group A animals (average, 0.256 g.) were distinctly larger than those of Group B (average, 0.182 g.), and the average total adrenal lipid per 100 g. of guinea pig was 50% more for Group A than for Group B (23.3 mg. and 15.5 mg., respectively).

Total Carcass Lipids. The carcasses, after removal of lungs and viscera, were chopped and pooled by groups. The same method of extraction was used as was employed for the smaller tissues. In spite of the great loss in weight of the animals in the deficient group, the weights of carcass lipids per 100 g. of carcass were not significantly different for the two groups (4.33 g. for Group A and 4.89 g. for Group B). Phospholipid contents of the body lipids were essentially the same for both groups. The fatty acid compositions of the acetone-soluble lipids of the two groups were not significantly different (13).

Analyses of Extracted Lipids

Phospholipid Analyses of Liver Lipids. Acetone separations of the individual liver lipids were made according to the method described by Bloor (14). Aliquots of the material insoluble in cool, dry acetone containing $MgCl_2$ were used for phospholipid and phosphorus determinations.

A colorimetric oxidative method was developed for the determination of the amount of phospholipid. In order to standardize this procedure, small aliquots of purified phospholipid (0.1 to 1.0 mg.) were oxidized with 10 ml. of a seven-to-one mixture of $N/8$ potassium dichromate and $M/10$ silver iodate in concentrated sulfuric acid at $100^\circ C$. for thirty minutes. A comparison with blanks was made in an Evelyn photoelectric colorimeter using a color filter (6600 Å). K values were calculated for each of the readings of the known solutions of phospholipids using the following relationship:

$$K = \frac{\log 100 - \log \text{galvanometer reading}}{\text{amount of phospholipid}}$$

The average K value obtained for the oxidation of purified phospholipid and used for subsequent determinations was 0.3065. The aliquots containing unknown amounts of phospholipid were oxidized in the same manner, and the amount of acetone-insoluble material was calculated from duplicate or triplicate determinations which agreed within a range of 3%. The average weight of phospholipid per 100 grams of liver lipid was somewhat higher for the Group A animals than for Group B animals (33.8 ± 1.9 and 26.8 ± 2.2 , respectively).

The phosphorus contents of each of the acetone insoluble lipids was determined by the method of Fontaine (15), the average being $4.0 \pm 0.1\%$. They were relatively free from other lipid material.

Cholesterol Analyses of Liver and Adrenal Lipids. The method used for the separation of free and total cholesterol from liver lipids and total cholesterol from adrenal lipids was essentially that described by Kelsey (16). The Liebermann-Burchard reaction was used on the separated cholesterol and the developed color was compared by means of the Evelyn photoelectric colorimeter with the color obtained when standard amounts of highly purified cholesterol (m.p. 148.5° C.) prepared by recrystallization and debromination of cholesterol dibromide were used. Although cholesterol analyses were made on individual lipids, only the averages are shown in Table II.

TABLE II

Cholesterol Analyses of Livers and Adrenal Lipids

	Groups	
	A	B
Number of animals used	25	22
Total cholesterol		
Per cent of liver lipid	5.8±0.3	6.9±0.4
Per cent of adrenal lipid	8.3±1.1	6.9±0.6
Free cholesterol		
Per cent of total cholesterol of liver lipid	85.1±1.5	78.1±2.3

Combined Liver Lipid Analyses. The phospholipids from all the livers of each group were combined after the above analyses were completed; they were saponified, and the non-saponifiable material was extracted. Iodine values and saponification equivalents of the purified free fatty acids were determined and are recorded in Table III. The acetone-soluble liver lipids were also combined and subjected to the same procedure and the results are shown in the same table.

TABLE III

	Groups	
	A	B
Free fatty acids of phospholipid		
Iodine value	75.0	32.7
Saponification equivalent	276.0	271.6
Free fatty acids of acetone-soluble lipid		
Iodine value	100.9	87.7
Saponification equivalent	278.5	272.4

DISCUSSION

This study, designed to clarify the uncertainty concerning the effects of avitaminosis C and inanition on hepatic fat in guinea pigs, indicated

that the gross amount of fat in the livers of animals developing scurvy while being fed a scorbutogenic diet was not significantly different qualitatively or quantitatively from the fat in the livers of their mates which were fed the same basal diet supplemented with ascorbic acid. Although the average amount of lipid in the livers of the two groups of animals was practically the same, there appeared to be an increase in the weight of the livers of the scorbutic pigs in spite of the fact that the body weights of these pigs showed more than 30 per cent decrease from maximum weight. The adrenal glands of the scorbutic animals showed a similar, but a relatively much larger, increase in weight as noted by Michaud (17). However, Svirbely (18) found the reverse to be true.

In no instances did the animals of the control group consume more feed than their paired mates in the deficient group. Therefore, any inanition effect, except perhaps poorer utilization of consumed food by the scorbutic animals, would be offset in the control group; and any difference in fat metabolism by the two groups could be ascribed to the effects of scurvy. Since no extremely fatty livers developed in any of the animals, the overall effects of inanition upon the hepatic fat content had not become apparent at the time the animals were killed. This fact is not in agreement with previous reports that inanition will cause fatty infiltration of the liver.

Although the animals of Group B were virtually without food for the last few days of life, no fatty livers developed strictly because of inanition. However, if the fasting time had been lengthened, it is entirely possible that the liver lipids of the control animals might have shown an appreciable increase. The percentage of fat in the control animals was extremely variable as noted by Best and Ridout (19). This fact might be accounted for by different degrees of inanition. It is also recognized that the effects of vitamin C deficiency might vary with respect to fatty livers at different stages of the onset of scurvy.

The only other noticeable effects of the scurvy were the relatively higher iodine values of the free fatty acids of both the liver phospholipids and liver acetone-soluble lipids from the deficient animals than from the control animals. What importance can be attached to this fact is not known, but the result is the reverse of the findings of DeCaro and Giani (20).

It is interesting to note that the carcass lipids expressed as per cent of carcass weight were practically the same for the two groups of

animals even though the deficient animals had lost an average of 30.5% of their maximum weight before death. The control animals lost only 12.4% of their maximum weight.

SUMMARY

1. The effect of avitaminosis C on fat metabolism in guinea pigs was studied using a paired feeding technique to rule out the effect of inanition which always accompanies the onset and the development of scurvy.

2. No significant difference was noted in the gross amounts of liver lipids, adrenal lipids, or carcass lipids in the scorbutic and control animals. Only one case of serious hepatic injury was noted among the vitamin C-deficient guinea pigs.

3. There were no significant changes in the phospholipid content of livers or cholesterol content of livers accompanying the development of scurvy.

4. The cholesterol content of adrenal lipids was not appreciably altered by the effects of scurvy.

5. The livers and adrenals of the scorbutic guinea pigs were significantly larger than were the livers and adrenals of the control animals.

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Component Fatty Acids of Guinea Pig Body Fat¹

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INTRODUCTION

Extensive data are available on the fatty acid composition of animal body fats (1) particularly with respect to the depot fat of rats fed various diets (2). However, there has been no comparable information concerning the fatty acid composition of the body fat of another widely used laboratory animal, the guinea pig. An opportunity to obtain such data presented itself in connection with another study (3).

METHODS AND RESULTS

Fats for the present study were obtained from two groups of animals which had been fed known diets on a paired feeding experiment for several weeks before death. The Group A animals had received only a scorbutogenic diet and were in the last stages of scurvy at the time of sacrifice. Group B animals were allowed to consume no more of the diet than their paired mates in Group A, but the diet of the second group was supplemented with ascorbic acid (3). No indications of scurvy were present in the latter animals, but the first stages of inanition had become apparent.

Preparation of Methyl Esters and Distillation. The lipid material was removed from the minced carcasses with ethyl alcohol and ether. After the phospholipids had been separated according to the method of Bloor (4), the acetone-soluble material was converted to soaps by saponification with alcoholic potassium hydroxide. The solvent was removed *in vacuo* on a steam bath and the soaps were taken up in distilled water. After acidification with 50% sulfuric acid, the free fatty acids were extracted with ethyl ether and washed free of water-soluble impurities. The ether solution of fatty acids was dried; ether was removed, and the fatty acids were then converted to the

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methyl esters using redistilled methanol with sulfuric acid as the catalyst. After a series of washings with potassium carbonate solution and distilled water to remove unesterified fatty acids and mineral acid, the neutral methyl esters were fractionally distilled at reduced pressure through a packed column (5) which had been shown to give sufficient separation to allow calculation of known mixtures to within 5% of any component present (6). The distillation data for the esters prepared from Group B animals are shown in Table I.

TABLE I
Analyses of Methyl Ester Fractions of Group B Carcass Lipid

Fraction	Weight g.	Iodine value (Wijs)	Saponifi- cation equiva- lent	Methyl octadeca- dienoate per cent	Methyl octadeca- trienoate per cent	Methyl eicosatet- raenoate per cent	Refractive Index $n_D^{35.0}$
B-21	1.616	13.7	235.1				1.4336
B-22	1.197	15.1	254.1				1.4352
B-23	5.117	10.5	267.8				1.4361
B-24	1.546	15.7	269.1	3.2	0.2		1.4368
B-25	3.855	78.3	287.0	26.1	1.2		1.4452
B-26	3.361	109.2	292.8	32.1	1.7		1.4487
B-27	5.771	107.8	294.5	33.0	1.8		1.4487
B-28	5.332	105.0	294.9	29.1	1.7		1.4486
B-29	4.646	101.4	296.3	26.1	1.8		1.4483
B-30	2.672	90.8	297.3	22.6	2.8		1.4481
B-31	1.437	121.7	311.3			10.5	1.4580
B-32	1.247	113.3	326.1			18.6	1.4462
B-33	4.479	56.3	981.1*			11.5	
B-33			363.1*				

* After removal of non-saponified material.

Analyses of Distillate Fractions. Iodine values, saponification equivalents, and the results of spectrophotometric studies on the isomerized soaps are given in Table I for the methyl ester fractions from Group B fat. The unsaturation in the ester fractions whose mean molecular weights were less than the molecular weight of the C_{18} esters was assumed to have been due to C_{14} and C_{16} unsaturated esters and was calculated as such. Insufficient material was available for accurate identification of these two probable components. The percentage of methyl octadecadienoate, octadecatrenoate, and eicosatetraenoate were calculated from the spectral absorption of the high temperature isomerized soaps at 234 $m\mu$, 270 $m\mu$, and 304 $m\mu$ respectively (7). The analyses of the methyl ester fractions resulting from the distillation of

the Group A esters were very similar to those shown for the Group B esters.

In Fig. 1 there are shown typical absorption curves for the isomerized soaps from three ester fractions of the Group B analysis. The lower

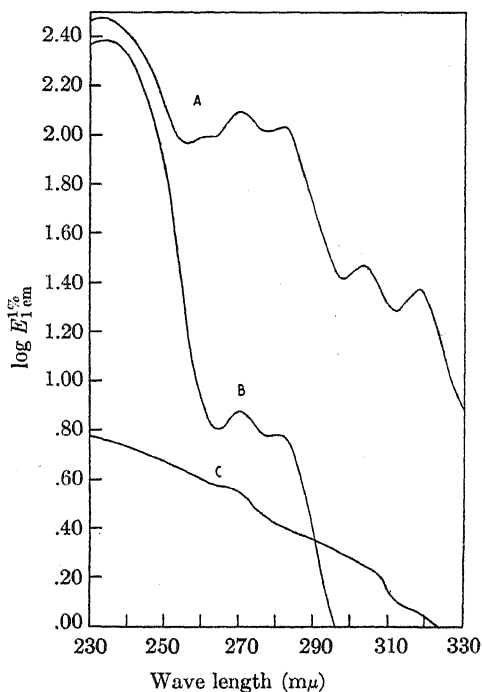


FIG. 1

Spectral Absorption Curves of the Soaps Resulting from the Isomerization of Several Methyl Ester Fractions

A, B-31; B, B-28; C, B-22

fractions, represented by curve B-22, which contain no C₁₈ esters according to the saponification equivalents, show only atypical, general absorption. The fractions, represented by curve B-28, containing esters of molecular weight no higher than C₁₈ esters have specific absorption peaks at 234 mμ and 270 mμ which represent conjugated diene and triene material, respectively. Fractions containing C₂₀ esters show absorption for conjugated tetraene material as well as

conjugated diene and triene material as indicated in Curve B-31. The tetraenoic ester was all calculated as eicosatetraenoic acid and all the remaining high molecular weight esters were calculated as " C_{20} " acid. The specific absorption coefficients used in these calculations were furnished by Beadle and Kraybill (8).

A summary of the component fatty acids of the acetone-soluble body fats from the two groups of guinea pigs is presented in Table II.

TABLE II

Component Fatty Acids of Acetone-soluble Carcass Lipids of Guinea Pigs

Acids	Weight percentage		Molar percentage	
	Group A	Group B	Group A	Group B
Lauric	—	1.1	—	1.5
Myristic	3.7	5.3	4.5	6.4
Palmitic	22.7	19.4	24.3	20.6
Stearic	7.3	5.7	7.1	5.5
Tetradecenoic	.4	0.8	0.5	1.0
Hexadecenoic	1.7	2.1	1.8	2.2
Octadecenoic	34.2	36.2	33.3	34.9
Octadecadienoic	18.4	18.8	18.0	18.3
Octadecatrienic	2.0	1.2	2.0	1.2
Eicosatetraenoic	2.4	2.4	2.2	2.3
Other C_{20} and C_{22} acids, "as C_{20} "	7.2	7.0	6.3	6.1

No gross differences in fatty acid composition are apparent between the fats from the animals which had scurvy and those which were free from scorbutic symptoms. However, it is not impossible that the effects of inanition which had been imposed upon all the animals may have served to equalize any minor differences in composition caused by avitaminosis C. Although the Group A animals had lost a much higher percentage of their body weights before death than had the animals of the control group, the lipids expressed as percentage of body weight were not significantly different, and no effect upon fatty acid composition is evident from the greatly different losses in body weight.

SUMMARY

Fatty acid analyses were made on the acetone-soluble carcass lipids of scorbutic guinea pigs and of their paired fed controls receiving ascorbic acid. The component fatty acids of these lipids were found to be very similar.

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Synergistic Effect of Penicillin and Anti-Pneumococcus Serum in the Experimental Pneumococcus Infection of Mice

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INTRODUCTION

In the course of experimental work on combination therapy with penicillin and other anti-pneumococcal drugs, which was instigated by the recent studies of Ungar (1) on the synergism of penicillin and sulfapyridine in streptococcus and staphylococcus infections, a most striking combination effect of penicillin and anti-pneumococcus serum was observed.

Methods

The experiments were carried out in groups of 5 to 20 white mice of 16-20 g.

Pneumococcus strains. Two strains of pneumococci were used, both obtained from the American Type Culture Collection. The strains, No. 6301 (type 1) and No. 6302 (type 2), were passed through mice regularly. Intra-abdominal infection with 0.3 cc. of a 10^{-6} dilution of overnight serum broth culture was used in all these experiments. This dose represents approximately 1000 MLD, and killed untreated control animals within 30 to 48 hours.

Anti-pneumococcus sera. The type 1 anti-serum was rabbit serum (Lederle, series MC 8/2/43, unpreserved). The protecting dose, given subcutaneously 3 hours before the intra-abdominal infection with 1000 MLD of Pn. 6301 was 0.5 cc. of a dilution 1-40,000. In these experiments, 0.5 cc. 1-100,000 was injected.

The type 2 serum was a commercial anti-serum from rabbits (Lederle 184H184Z). The dose which protected all mice infected intra-abdominally with 1000 MLD of Pn. 6302 was 0.5 cc. 1-100,000, given subcutaneously 4 hours before the infection. In these experiments, 0.5 cc. of a 1-300,000 dilution was injected.

Penicillin. The penicillin (CS) used in these experiments was a sodium salt of moderate activity (100 units/mg.) prepared by the chemical research laboratories. The active dose which protected 60 to 100% of mice infected intra-abdominally with 1000 MLD of Pn. 6301 or 6302 was 50 to 100 units per 20 g. mouse if given subcutaneously shortly after the infection. A single treatment with 1 mg. (= 100 units) had the same effect as a double treatment with 0.5 mg. (total: 100 units)

and with 0.25 mg. (total: 50 units). In these experiments, doses of 0.3 and 0.2 mg. per 20 g. were given, corresponding to 30 and 20 units.

Combination experiments. Treatment with anti-serum was administered 4 hours before the infection. The subcutaneous treatment with penicillin followed the intra-abdominal infection immediately. One single treatment was given. Control groups with anti-serum alone and penicillin alone as well as a group of untreated mice were included in every experiment.

EXPERIMENTAL

The results of the experiments, tabulated on Table I, demonstrate that the combination of inactive doses of anti-pneumococcus serum type 1 and type 2 with subtherapeutic doses of penicillin exerted a striking synergistic effect. Anti-serum alone and penicillin alone were practically inactive, but 90 to 100% of all mice survived if both agents were given.

TABLE I

Combination of a Single Subcutaneous Treatment with Penicillin CS with Type 1 and Type 2 Anti-Pneumococcus Serum in Infections with Pneumococcus Type 1 and Type 2

Pneumo- coccus	Type	Antiserum* Dose	Penicillin CS†	Mice	Number of Survivors
6301	1	1-100,000	—	20	3
(type 1)		1-100,000	0.2 mg.	20	20
		—	0.2 mg.	20	0
		—	—	10	0
6302	2	1-300,000	—	5	0
(type 2)		1-300,000	0.3 mg.	10	9
		1-300,000	0.2 mg.	10	9
		—	0.3 mg.	5	0
		—	0.2 mg.	5	0
		—	—	8	0

* Subcutaneous injection 3 hours before the infection.

† Subcutaneous injection shortly after the infection.

DISCUSSION

The combination of anti-pneumococcus serum and penicillin, described in the present paper, is the strongest synergistic activity observed in pneumococcus experiments so far. In infections with hemolytic streptococci, one obtains similarly high survival rates if penicillin and different sulfonamides are combined, as has been shown by

Ungar (1) and by Soo-Hoo (2) in this laboratory. Recent experimental work with pneumococci, carried out with L. C. Lafferty in this laboratory, showed, however, that the combination of penicillin + sulfapyridine is by no means very satisfactory, while penicillin + sulfadiazine gave better results which are, nevertheless, not so striking as those obtained with anti-pneumococcus serum. Anti-pneumococcal agents of the quinine group: such as, ethylhydrocupreine (Optochin) did not show any synergistic activity whatever. These findings suggest that the success of combined therapy with penicillin depends largely on the agent selected for the combination. From our experiments, we should draw the conclusion that the combination with specific anti-serum is particularly favorable in pneumococcus infections. The mechanism of this synergistic activity warrants further investigation.

SUMMARY

Subtherapeutic doses of specific anti-pneumococcus serum (type 1 and type 2) combined with inactive doses of penicillin sodium salt gave a highly effective synergism in experimental infections with type 1 and type 2 pneumococci. The rate of survival of the animals which received the combined treatment was 90 to 100%.

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Growth, Reproduction, and Lactation in Mice on Highly Purified Diets, and the Effect of Folic Acid Concentrates on Lactation¹

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In contrast to the extensive investigations on the nutritional requirements for growth, reproduction, and lactation which have been carried out on rats maintained on highly purified diets, very few data are available for the mouse. Woolley (1) found that mice grew normally on a purified diet containing the vitamin B factors supplemented with inositol. Troescher-Elam and Evans (2) were unable to obtain normal growth in mice on artificial rations. Similar findings were reported by Foy and Cerecedo (3), and by Sandza and Cerecedo (4). Rogers, *et al.* (5) managed to raise three generations of mice on a synthetic diet, but lactation was poor, and the average number of young per litter weaned was small. They found it necessary to feed a rice polish filtrate in addition to the crystalline vitamins. Attempts to raise another strain of mice on the same diet were unsuccessful. Foster, *et al.* (6) succeeded in raising four generations of mice on a highly purified diet. However, the weaning weights and the growth of the mice were inferior to those of the controls. The subnormal growth became more noticeable as the number of generations increased until in the F₃ generation the weanling mice died if separated from the mother. The diet used by these workers contained fibrin as a source of protein instead of casein, which makes it difficult to evaluate their results.

We have been able to raise mice through four generations on highly purified diets and have obtained excellent growth throughout the

¹ The substance of this paper was presented before a session of the Division of Biological Chemistry, 106th Meeting of the American Chemical Society, Pittsburgh, September 1943. This investigation was supported by a grant from the John and Mary R. Markle Foundation.

experimental period. However, the size of the litters weaned and the percentage of litters weaned were below normal. Also, a condition which we observed in lactating rats kept on artificial rations (7) manifested itself in mice, namely, a loss in weight of the mother during lactation.

Several supplements were fed to the mice to determine whether lactation could be improved. Those that showed a marked beneficial effect were brewer's yeast and two concentrates of "folic acid."

EXPERIMENTAL

To obviate any possible strain variations in these studies, three strains of mice were used. They were the Rockland albino and black strains, and our own albino strain. Two basal diets were used (Table I). The mice were placed on the synthetic rations when 21-25 days old and weighing 8-12 g. Records were kept of the food intake during growth, reproduction, and lactation. The animals were mated when 80-90 days old.

TABLE I
Composition of the Basal Diets

Component	Diet	
	R-5	C-28
Purified Casein (Smaco)	30	25
Salts (Osborne and Mendel) ^{1a}	5	5
Ruffex ^{1b}	2	2
Lard	5	5
Crisco	10	10
Sucrose	48	53

Supplements for diet R-5 were per kilogram: thiamin, 20 mg.; riboflavin, 20 mg.; pyridoxin, 20 mg.; calcium pantothenate, 40 mg.; choline chloride, 500 g.; α -tocopherol, 20 mg.; vitamin A and D concentrate, 40 mg.

Supplements for diet C-28 were per kilogram: thiamin, 10 mg.; riboflavin, 10 mg.; pyridoxin, 10 mg.; calcium pantothenate, 100 mg.; choline chloride, 1.5 g.; α -tocopherol, 40 mg.; carotene,^{1c} 2 mg.; vitamin D (Drisdol),^{1d} 125 μ g.

In studying the effect of the folic acid concentrates, these materials were incorporated into the basal diets. One of the concentrates was prepared in this laboratory from Wilson's Liver Fraction L² according to the method of Hutchings, *et al.* (8). This was a crude preparation where only one adsorption and elution of the factor

^{1a} The amount of MnSO_4 in the salt mixture was doubled.

^{1b} α -cellulose preparation obtained from Eimer and Amend, New York.

^{1c} The carotene used was a preparation containing 90% β - and 10% α -carotene. It was obtained from the S. M. A. Corporation, Chicago.

^{1d} Obtained from Winthrop Chemical Co., New York.

² Obtained from The Wilson Laboratories, Chicago.

on Norite A had been made. It showed a folic acid activity of "potency 10"³ as compared with a sample of Wilson Liver Fraction B,⁴ established as a standard of potency one (9). One and one-half grams of this crude material was mixed with one kilo of the basal ration, C-28 or R-5, to prepare the CF-28 or RF-5 diets. The amount of folic acid of "potency 40,000" (9) that one kilo of such a diet contained was 375 μ g. or "0.375 milligram-units," where one mg. of folic acid of "potency

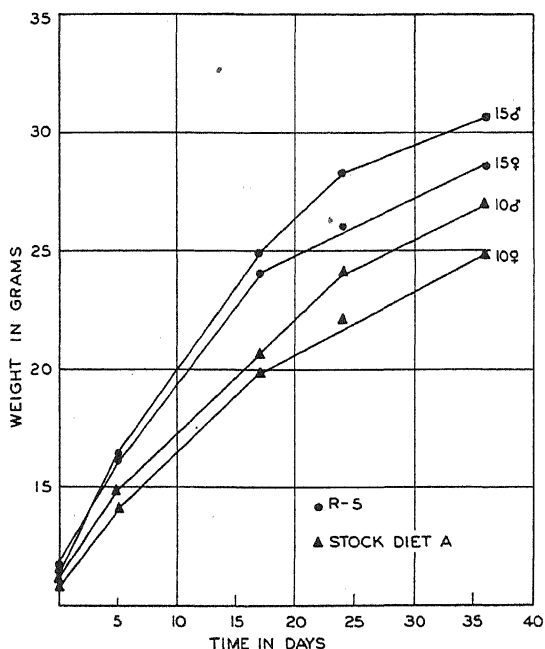


FIG. 1

Average Growth Curves, Beginning at the Time of Weaning, of Second Generation Mice Fed Diet R-5, and of Controls Kept on Stock Diet A

The number at the end of each curve represents the number of mice used in each experiment.

40,000" has been designated by Williams (10) to contain "one milligram-unit." The other folic acid concentrate which we used had a potency of 5000 and was generously supplied by Dr. R. J. Williams. Twenty-five mg. of this material was incorporated into one kilo of the R-5 diet to make up the R-11 diet. This ration

³ Through the courtesy of Dr. R. J. Williams the biological activity of this preparation was tested at the University of Texas.

⁴ We are indebted to Dr. David Klein of The Wilson Laboratories for this material.

contained 3.125 mg. of folic acid of "potency 40,000" or "3.125 milligram-units" per kilogram of diet.

The growth of the three strains of mice on the purified diets was excellent throughout several generations. In fact, it was superior to that of the controls fed the stock diet⁶ (stock diet A). Growth curves of one group of mice kept on diet R-5, and of another kept on stock diet A are shown in Fig. 1. There was no improvement in the growth of the mice when fed the diets containing folic acid (diets RF-5, CF-28, and R-11). On the basis that the mice ate on an average 3 g. of the diet daily, the amount of folic acid of potency 40,000 ingested per day was slightly more than 1 μ g. in the case of the mice fed diets RF-5 and CF-28, and approximately 10 μ g. for those fed diet R-11. In this connection, it is of interest that Mitchell, *et al.* (11) reported increased growth in rats fed daily a supplement of 50 μ g. of a folic acid preparation.

Upon mating the mice on the basal diets (C-28 and R-5), reproduction and lactation were obtained in the two albino strains. However, the number of litters weaned and the average size of the litters did not compare favorably with those found with mice given stock diet A (Table II). Thus we have here two purified diets which

TABLE II
Data on Reproduction and Lactation

Strain	Diet	No. of Females	Litters born	Litters weaned	Per cent of litters weaned	No. of young per litter (average)
Our own	R-5	18	15	7	47	5.0
Our own	RF-5	10	10	8	80	6.4
Our own	R-11	5	5	4	80	5.0
Our own	C-28	15	10	4	40	4.0
Our own	CF-28	10	8	7	87	4.0
Rockland albino	R-5	14	13	6	46	2.7
Rockland albino	RF-5	6	5	5	83	6.2
Rockland black	R-5	6	5	0	—	—
Rockland black	RF-5	5	5	1	20	8.0
Rockland black	C-28	7	5	0	—	—
Rockland black	CF-28	11	9	2	22	3.5
Rockland black	R-11	8	7	3	46	3.0
Our own	Stock diets A and B	25	20	15	75	6.4
Rockland black	Stock diets A and B	16	12	8	66	5.0

were superior to the control diet for growth but inferior for reproduction and lactation. Another indication of the inadequacy of the basal diets was the loss in weight exhibited by most of the lactating mice. Occasionally the mice would show a gain in weight in rearing their first litter if there were two or three young to a litter,

⁶ Purina dog chow.

but these were exceptions. The controls on stock diet A did not show this loss in weight during lactation except in isolated cases.

Lactation was a complete failure in the case of the Rockland black mice kept on the basal diets (Table II). This was not unexpected, since we found it difficult to

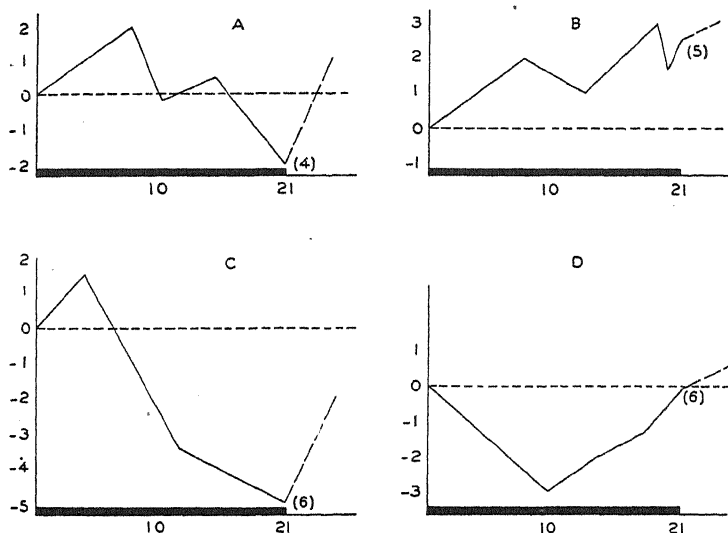


FIG. 2

Fluctuations in the Weight of Mice during Lactation

Two representative examples of lactating mice on diet R-5 (graphs A and C) are contrasted with two mice (graphs B and D) showing the typical effect obtained with diet RF-5 during lactation. The dotted line parallel to the abscissa in each graph refers to the weight of the mother (in grams) at the time of littering. That part of the curve above this line indicates a gain in weight (in grams) during the 21 day period of lactation (represented by a heavy black line), and that segment below this line signifies a loss in weight during the same period. Note that whereas the curves in graphs A and C show a steady decline as lactation approaches the 21st day, those in graphs B and D show a positive inflection during the second half of the lactation period. The number in parentheses in each graph refers to the number of young weaned.

obtain litters from this strain even on stock diet A. The percentage of litters weaned, which was below 50% on this diet, was improved when the animals were given, in addition to stock diet A, a ration consisting of ground oats, 50; brewers' yeast,⁶ 10; whole milk powder (Klim), 15; and NaCl, 1.2 (stock diet B). They were also fed lettuce and fresh liver once a week.

⁶ Brewers' Yeast Powder, supplied by Mead Johnson and Co., Evansville, Indiana.

In an effort to improve lactation and prevent the loss in weight of lactating mothers, diets RF-5, CF-28, and R-11 were given to the mice (Table II). The effect of these rations on lactation was marked. In the case of the white mice, lactation was as good as that of the controls on stock diet A. The percentage of litters weaned was doubled, and the size of litters weaned was substantially increased. Also, the majority of the lactating mice gained in weight while rearing their young. The loss in weight of mice during lactation on diet R-5 and the beneficial effect of a diet containing a folic acid concentrate (diet RF-5) are shown in Fig. 2. Upon separation of the mother from the young, there was a rise in body weight. The significance of this abrupt gain in weight immediately after separation from the litter is obvious. Lactating mice on the basal diet were breaking down their body tissue in an effort to satisfy the demands of the suckling young, since the diet was not adequate for normal lactation. Upon separating the mother from its offspring, the strain was removed, and an immediate restoring of body reserves began. The food intake of such mice after separation from their young was high, 4-5 g. daily. This amount gradually decreased to the normal level of 3 g. in about a week.

Lactation performance in the case of the black mice on the folic-acid-fortified diets was poorer than that of the albino mice on the same diets. However, the effect of the folic acid concentrates on the black mice is specially noteworthy, since we failed completely in our attempts to obtain litters from this strain on the basal rations. Investigations are continuing with this strain, using diets richer in folic acid, to determine if this substance is the limiting factor in lactation.

Other supplements which were given in addition to the basal diets in an effort to improve lactation were as follows: Biotin, 0.05 μ g./day, yeast nucleic acid, 9 mg./day, *p*-aminobenzoic acid plus inositol, 250 mg. of each added to one kilo of diet R-5, Ryzamin B,⁷ 25 mg./day, and brewers' yeast,⁸ 100 mg./day. Of these only the brewers' yeast and, to a lesser extent, Ryzamin B gave improved lactation.

DISCUSSION

The growth of the mice on the basal diets (diets R-5 and C-28) was excellent. These rations contained just five of the B complex vitamins in crystalline form. These findings do not support the view of Woolley (12), who stated that mice required inositol for normal growth. Neither do they substantiate the ideas of Troeschel-Elam and Evans, and of Sandza and Cerecedo, who believed that some unknown factor present in liver was necessary to obtain normal growth of mice on purified rations. The excellent growth obtained in our experiments may perhaps be attributed to the larger quantities of vitamins or to the nature of the fat supplied by our artificial rations, or to both of these factors.

⁷ Generously supplied by Burroughs Wellcome and Co., Inc., New York.

⁸ Brewers' yeast powder, strain K. We are indebted to Dr. E. H. Harvey of Anheuser-Busch, Inc., St. Louis, for a generous supply of this material.

Lactation of the mice on the basal diets was poor as compared with that of the mice on the stock diet. The addition of a crude preparation of folic acid of potency 10 greatly improved lactation. There is a possibility that such a crude concentrate was supplying something else besides folic acid, and which was having an effect on lactation. With a view to determining whether folic acid was the sole factor in improving lactation, a folic acid concentrate of potency 5000 was tested. One kilo of the diet containing this concentrate (diet R-11) contained more than 8 times the quantity of folic acid present in an equivalent amount of diet RF-5. By cutting down the amount of the concentrate which we added to one kilo of the basal diet sixty-fold, from 1.5 g. to 25 mg., we eliminated to a great extent the possible presence of unknown factors in the concentrate. It has been shown that on diet R-11 black mice were able to rear litters. The effect of the folic acid was conclusive, since this particular strain of mice always failed to rear litters on the basal diets. Lactation was not normal, however, since several litters were destroyed when one or two weeks old. This points to a need for larger quantities of the folic acid concentrate. The possibility that some other factor or factors still are needed along with folic acid is, of course, not completely ruled out.

SUMMARY

1. Three strains of mice have been reared through several generations on highly purified diets.
2. Growth of the mice on the artificial rations was excellent and was superior to that of the controls kept on the stock diet. However, the control mice gave better results as far as reproduction and lactation were concerned.
3. The poor lactation performance of mice on the artificial rations was considerably improved by the addition of a folic acid concentrate to the rations.

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The Determination of Phenylalanine in Proteins

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INTRODUCTION

The first method for the determination of phenylalanine other than by isolation is that of Kollmann (1) whose procedure involved the formation of benzoic acid by the oxidation of phenylalanine with potassium bichromate in an acid medium. The benzoic acid was extracted with ether and finally dried and weighed. Large amounts of protein (15–20 g.) are required and the values obtained are, according to Kollmann, minimal. In 1930 Brazier (2) introduced a method for the determination of a number of amino acids based upon the different degrees of solubility of their copper salts in solvents such as water, methyl alcohol, and ethyl alcohol. The copper salts of leucine, aspartic acid, and phenylalanine were insoluble in water and were separated by appropriate means. Baptist and Robson (3) were unable to isolate pure phenylalanine by the method of Brazier. They, in turn, suggested a procedure whereby phenylalanine was salted out together with leucine from a hydrochloric acid hydrolyzate of the protein. The phenylalanine was separated from the leucine by precipitation as the picrolonate. More recently Martin and Synge (4) and Gordon, Martin, and Synge (5) suggested a procedure for the separation of phenylalanine based upon partition chromatography.

Kapeller-Adler (6) proposed a colorimetric method involving the nitration of phenylalanine and the subsequent development of a violet color by the addition of hydroxylamine in the presence of ammonium hydroxide. This method was a variation of one early proposed by Mohler (7) for the detection of benzoic acid. The chemistry of the Kapeller-Adler reaction was studied by Block and Bolling (8) who found that the color was not due to the formation of either 3-nitroso-4-nitrobenzoic acid or 3-nitro-4-nitrosobenzoic acid as suggested by

Kapeller-Adler but rather to the formation of a derivative of diaci-*o*-dihydronitrobenzene or diaci-*o*-dinitrophenylalanine. Jervis, Block, Bolling, and Kanze (9) used the Kapeller-Adler reaction and found that filter 560 m μ would remove the interfering color produced by tyrosine and histidine. However they do not mention any interference from tryptophan which Kapeller-Adler believed to be destroyed during acid hydrolysis nor is this considered in a later paper by Block, Jervis, Bolling, and Webb (10) in which hydrolysis with 5 *N* NaOH is recommended, a procedure which does not destroy tryptophan. Knight and Stanley (11) found that the presence of tryptophan did interfere positively in this procedure. Erlenmeyer and Lipp (12) prepared *p*-nitrophenylalanine which they reduced with tin and hydrochloric acid to *p*-aminophenylalanine. Block and Bolling (8) using a nitration procedure similar to that of Kapeller-Adler (6) prepared 3,4-dinitrophenylalanine.

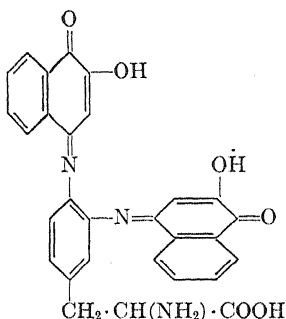
EXPERIMENTAL

Phenylalanine was nitrated by a procedure similar to that of Block and Bolling. The resulting 3,4-dinitrophenylalanine in acid aqueous solution was reduced by zinc powder, filtered, and combined with 1,2-naphthoquinone-4-sodium sulfonate as first employed by Böniger (13) with aniline. Over a long period of years in a study of the reaction of 1,2-naphthoquinone-4-sodium sulfonate with various compounds Sullivan (14) found the reaction of aminobenzene derivatives in an acid solution, with the formation of a red precipitate soluble in alcohol, to be highly specific since other types of compounds react with the naphthoquinone only on the alkaline side.

Chemical Structure of the Colored Compound

412.5 mg. of *l*-phenylalanine were dissolved in 3 g. of concd. sulfuric acid and placed upon the water bath and 0.98 g. of barium nitrate was added. After remaining on the water bath for 20 minutes the solution was diluted to about 25 cc. with water and most of the sulfuric acid removed by the addition of a saturated aqueous solution of barium hydroxide. The barium sulfate was filtered off, washed, and to the deep yellow solution (about 100 cc.) there were added 5 cc. concd. hydrochloric acid and mossy tin. The solution was again placed upon the water bath and boiled until it was colorless and then filtered into a solution containing 1.4 g. 1,2-naphthoquinone-4-sodium sulfonate (2 mols). The precipitate which formed almost immediately was allowed to stand in the ice box overnight, and was then removed by centrifugation, washed 4 times with water, and finally dissolved in hot alcohol and

allowed to crystallize. The reddish brown solid was dried in a drying pistol with toluene as the boiling liquid. A melting point determination was rather difficult to make because the sample was so dark. However it began to swell and become liquid between 175 and 180°. Nitrogen analysis showed 8.03% N, theory for $C_{23}H_{21}O_6N_3$ 8.28% N. A similar compound was prepared by reduction of 3,4-dinitrobenzoic acid and coupling with the naphthoquinone sulfonate and on analysis it showed 5.79% N, theory for $C_{27}H_{16}O_6N_2$ 6.03% N. The difference in nitrogen content of the two samples is sufficient to indicate that the compound is a product of diamino-phenylalanine and 1,2-naphthoquinone-4-sodium sulfonate of the following formula.



Possible Interference of Other Amino Acids

Kapeller-Adler (6) found several amino acids interfering with the estimation of phenylalanine following nitration and treatment with hydroxylamine in ammoniacal solution. Accordingly 5 mg. each of these amino acids, tryptophan, histidine, and tyrosine, respectively, were placed in glass evaporating dishes and 2.0 cc. of the nitrating mixture (10 g. sodium nitrate dissolved in 100 cc. concd. sulfuric acid) were added to each. The mixtures were placed on the water bath for 20 minutes and then 10 cc. water were added to each dish and about 100 mg. of powdered zinc. The solutions were stirred and allowed to remain on the water bath for 15 minutes, removed and kept for 30 minutes at room temperature. Each solution was then diluted to 20 cc. with water and filtered and tested colorimetrically. To 1.0 cc. of the filtrate was added 1.0 cc. of a 0.25% aqueous solution of 1,2-naphthoquinone-4-sodium sulfonate. After standing for 15 minutes at room temperature, 3 cc. of 95% ethyl alcohol were added. As a blank 1 cc. of water was similarly treated. Tyrosine and histidine remained the color of the blank while both tryptophan and phenylalanine gave a red color. However interference from tryptophan was abolished by the use of a 2.5% solution of potassium permanganate which Kapeller-Adler used to remove tyrosine and found to be non-destructive of phenylalanine.

Reproducibility of Results and Sensitivity of the Method

A phenylalanine solution was prepared by dissolving 100 mg. of analytically pure l-phenylalanine in 100 cc. of 0.5 N sulfuric acid. Four aliquots of 2.5 cc. each of this solution were nitrated, reduced and diluted to 20 cc. as given above. Four separate

determinations were made upon 1.0 cc. (0.125 mg. phenylalanine) aliquots from each of the 4 resulting filtrates. The colored solutions were read in a Klett-Summerson photoelectric colorimeter with a 54 filter. The reading of the naphthoquinone blank was subtracted from all values. The results of the 16 separate determinations showed an average of 0.122 mg. with a standard deviation of 0.004 mg. Dilutions of the several filtrates were made so that 1 cc. of the final solution before colorimetric analysis contained from 0.0062 to 0.244 mg. and the colorimetric reading varied from 11 for the lowest concentration to 440 for the highest concentration. The values obtained from colorimetric readings fall upon a straight line so the color produced is directly proportional to the concentration. The smallest amount of phenylalanine that can be estimated is approximately 6 micrograms.

Effect of the presence of other amino acids. To 2.5 cc. of the phenylalanine standard there were added 2.5 mg. of tryptophan and 2.5 mg. of tyrosine. A 2.5% solution of potassium permanganate was then added dropwise with stirring until a rose color persisted for at least one minute. The standard for comparison was 2.5 cc. of the phenylalanine standard solution. Both solutions were taken down to an oil on the water bath and the phenylalanine estimated as previously detailed. The recovery was 97.6% of the phenylalanine in the presence of the two amino acids.

A mixture was then prepared containing 5 mg. each of the following amino acids: cystine, methionine, leucine, isoleucine, arginine hydrochloride, glutamic acid, alanine, glycine, proline, and valine, and to it was added 2.5 cc. of the phenylalanine standard. Nitration and color development were conducted as previously described in this mixture and also in a similar mixture to which 0.6 cc. of the 2.5% KMnO_4 solution had been added. A standard solution of phenylalanine was run through the same procedure at the same time. The recovery of the phenylalanine was 96.2% in the presence of the potassium permanganate and 97.3% in the solution to which the potassium permanganate had not been added. It can thus be seen that none of these amino acids interfere with the determination of phenylalanine either in the presence or absence of KMnO_4 and that the potassium permanganate does not destroy the phenylalanine.

Other possible interferers containing the benzene ring might be *p*-aminobenzoic acid or benzoic acid itself. Phenylalanine added to a solution containing both of these compounds was estimated quantitatively. Finally a solution containing all of the possible interferers, 5 mg. each of benzoic acid, *p*-aminobenzoic acid, tyrosine, tryptophan, and histidine in addition to 2.5 mg. of phenylalanine in 2.5 cc. of 0.5 *N* sulfuric acid was prepared. The addition of 3.3 cc. of the permanganate solution produced a rose color that persisted for one minute. Eight determinations of phenylalanine in this mixture were made and the average recovery was 101.8% of the phenylalanine added. Phenylalanine, consequently, can be estimated in the presence of other amino acids and also in the presence of benzoic acid and *p*-aminobenzoic acid and should be determinable in a protein hydrolyzate.

Determination of phenylalanine in proteins. Three different methods of hydrolysis employing (1) 7 *N* sulfuric acid, (2) 20% hydrochloric acid, and (3) 5 *N* sodium hydroxide, were used for varying periods of time. For the two types of hydrolysis using acid the procedure was as follows: 200–500 mg. of the protein were hydrolyzed with 2.0 cc. of the acid, and 0.5 cc. of butyl alcohol was added to prevent foaming.

The flask, fitted with a ground glass reflux condenser, was immersed in an oil bath maintained at 125–130° and heated for the selected length of time. After cooling, the hydrolyzate with washings was poured into a 50 cc. graduate, diluted to 30 cc. with water and filtered. For analysis 6.0 cc. aliquots were placed in glass evaporating dishes and 2.5% KMnO_4 solution added, dropwise with stirring, until the rose color of the permanganate persisted for at least 1 minute, this required from about 0.1 cc. for gelatin to about 0.3 cc. for casein. The solutions were then placed on the water bath together with a standard solution, usually 2.5 cc. phenylalanine 1000 p.p.m. in 0.5 *N* sulfuric acid, and concentrated to an oil. After concentration 2 cc. of the nitrating mixture were added to each and the heating continued for 20 minutes. Then 10 cc. of water were added and about one decigram of zinc powder. The dishes were allowed to remain on the water bath for 15 minutes, removed, and kept at room temperature for 30 minutes. Each solution was then diluted to 20 cc. with water and filtered. The filtrates were used for colorimetric analysis. The hydrolyzates were usually divided into 4 aliquots, and there was always enough solution to make a number of determinations upon each aliquot. In case the sample size is less than 200 mg., the hydrolyzate can be divided into fewer aliquots. The procedure for colorimetric determination is the same as described in the section on possible interference of other amino acids. A Klett-Summerson colorimeter with filter 54 was always used. Two blanks are required, (1) to compensate for the slight color of the filtrate and (2) to compensate for the color of the 1,2-naphthoquinone-4-sodium sulfonate solution. The blank on the filtrate was prepared by adding 1 cc. of water to 1 cc. of the filtrate followed by 3 cc. of 95% ethyl alcohol. The colorimeter reading on this blank varies from 0 to 6. The blank on the naphthoquinone reagent was prepared by substituting 1 cc. water for 1 cc. of the filtrate. The readings of the two blanks are subtracted from the reading on each of the solutions.

The hydrolysis with 5 *N* sodium hydroxide was carried out by adding 2.0 cc. of 5 *N* sodium hydroxide and 0.5 cc. of butyl alcohol to 200–500 mg. of the protein. The heating was conducted at 125–130° as in the acid hydrolysis. The hydrolyzate was poured into a 50 cc. graduate, and with washing, made acid by the addition of 1.7 cc. of 14 *N* sulfuric acid, diluted to 30 cc. with water and filtered. After the addition of the proper amount of 2.5% permanganate solution to each aliquot the solutions were taken down to dryness on the water bath and the procedure from there on was identical with that described previously. Several determinations were made using 5 *N* sodium hydroxide for hydrolysis in a sealed tube. The sample, 25 mg., and 0.5 cc. of 5 *N* sodium hydroxide were sealed in a piece of glass tubing about 3 inches in length. The sealed tube was placed in an oven at 110° for 2 hours. The tube was then allowed to cool, broken, and the contents poured into a glass evaporating dish and the tube washed. To the solution was added 0.4 cc. 14 *N* sulfuric acid, and the procedure carried out as above described.

Five proteins were taken for analysis. Gelatin (Eimer and Amend sample), casein prepared by the method of Van Slyke and Baker (15), squash seed globulin prepared by the method of Vickery, *et al.* (16), zein prepared by L. S. Nolan and furnished by Dr. H. B. Vickery of the Connecticut Experiment Station, and egg albumin prepared by the method of Kekwick and Cannan (17). The results of the analyses upon these proteins, all corrected for moisture and ash, are given in Table I. Each

TABLE I
Phenylalanine Content of Proteins

Time hours	Method of hydrolysis	Protein				Squash seed globulin per cent
		Gelatin per cent	Casein per cent	Zein per cent	Egg albumin per cent	
2	7 N H ₂ SO ₄	1.71	3.89	2.19	3.29	2.74
	20% HCl	2.55	5.03	5.09	5.72	5.64
	5 N NaOH	2.37	4.97	6.49	5.67	5.69
	5 N NaOH sealed	2.29	5.37	6.41	5.92	5.82
4	7 N H ₂ SO ₄	2.42	5.69	5.79	6.06	4.82
	20% HCl	2.55	4.39	6.06	5.80	5.73
	5 N NaOH	2.38	4.73	6.56	5.80	5.77
6	5 N NaOH	2.39	5.71	6.62	6.18	5.82
8	7 N H ₂ SO ₄	2.45	5.46	6.74	6.00	5.30
	20% HCl	2.04	4.81	5.83	4.82	5.18
18	7 N H ₂ SO ₄	2.11	4.71	6.33	5.78	5.30
	20% HCl	1.59	3.93	5.52	5.84	4.66
	5 N NaOH	2.40	5.52	6.77	5.90	5.86

value in this table represents the average of at least 4 determinations upon the same hydrolyzate or 8 determinations on two different hydrolyzates.

DISCUSSION OF RESULTS

The values obtained by the three different methods of hydrolysis on each of the proteins are, in general, in good agreement. If the average value of the highest figure found for each protein by the three hydrolytic methods is calculated and that for the sodium hydroxide method is taken as 100% the values for the sulfuric acid and the hydrochloric acid methods are 97.5 and 93.7% respectively. Hydrolysis with 5 N sodium hydroxide yields a hydrolyzate which is almost colorless while the hydrolyzates produced by the two acids are always colored light yellow to tan. The two proteins known to contain no tryptophan, gelatin and zein, were run with and without the treatment with 2.5% KMnO₄ and the values were compared. In the case of zein the value omitting the permanganate was 102.3% of that when the permanganate was used, and in gelatin the value was 103.4%.

Although many trials in various fields of analysis of amino acids has shown that estimation of a free amino acid added to a protein is not a criterion of the goodness of a method it can be said that the recovery of phenylalanine added to gelatin was 100%, and when added to casein 98%.

In order to determine the reproducibility of the value upon a protein hydrolyzate, 4 separate hydrolyzates were prepared from squash seed globulin using 5 *N* NaOH and heating for 2 hours. Eight determinations were made on each hydrolyzate. The averages were $5.34 \pm .14$, $5.5 \pm .07$, $5.52 \pm .09$, and $5.61 \pm .06\%$. The average of the entire 32 values was $5.5 \pm .11\%$, and the spread from the highest to the lowest average is about 5%.

Gelatin. The highest value found was 2.45%. Fischer, Levene, and Aders (18) found 0.40%, Dakin (19) 1.4% by isolation methods while Kollmann (1) reported 0.2–0.29%; Kapeller-Adler (6) 0.93–1.27%, average 1.15%; and Block, Jervis, Bolling, and Webb (10) a maximum value of 2.6%. Gordon, Martin, and Synge (5) report 2 values for phenylalanine, 2.5 and 0.86%.

Casein. The highest value found was 5.71%. Kollmann (1) found 2.7–3.4%; Kapeller-Adler (6) 4.52–5.33%; average 5.00%; and Virtanen, Laine, and Toivonen (20) using the method of Kapeller-Adler found 5.4%. Block, Jervis, Bolling, and Webb (10) reported 5.8% as a maximum value. In 1940, Baptist and Robson (3) isolated 2.9%.

Zein. The highest value was 6.77%. Osborne and Clapp (21) isolated 4.9%, and Osborne and Liddle (22) isolated 6.6%. Dakin (23) claimed the isolation of 7.6% but offered no evidence of the purity of his isolated material. Baptist and Robson (3) isolated 5.1%.

Egg albumin. The highest value found was 6.18%. By isolation E. Fischer (24) obtained 2.5%; Abderhalden and Pregl (25) 4.4%; and Osborne, Jones, and Leavenworth (26) 5.07%. Three values have been obtained using the Kapeller-Adler method, 5.32% by Arnow, Burns, and Bernhart (27); 6.8% by Block, *et al.* (10) and 5.39% by Virtanen, Laine, and Toivonen (20).

Squash seed globulin. The highest values obtained were 5.82 and 5.86% with 6 and 18 hours hydrolysis by 5 *N* sodium hydroxide. No values for phenylalanine in this protein were found in the literature.

Two values were obtained upon *tobacco mosaic virus protein* which are not included in the table. The protein was the same sample previously analyzed by Hess, Sullivan, and Palmes (28). Six hours hydrolysis with 5 *N* NaOH gave 5.87% phenylalanine and 2-hours hydrolysis in a sealed tube gave 5.90% phenylalanine. Ross (29) using the procedure of Block, Bolling, Jervis, and Webb (10) reported 6.6% phenylalanine while Knight and Stanley (11) after correcting for the interference of tryptophan in this procedure found 6.0% phenylalanine.

SUMMARY

A method for the determination of phenylalanine has been described involving the formation of dinitrophenylalanine, its subsequent reduction to diaminophenylalanine and coupling in slightly acid solution with 1,2-naphthoquinone-4-sodium sulfonate to yield a red compound. The method has been applied to the determination of phenylalanine in casein, gelatin, zein, egg albumin, and squash seed globulin, following hydrolysis for varying lengths of time with 7 *N* sulfuric acid, 20% hydrochloric acid, and 5 *N* sodium hydroxide. The values obtained for each of the proteins by the three methods of hydrolysis were in good agreement. The colorimetric procedure described will detect 6 μ g. of phenylalanine per cubic centimeter.

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A New Method for the Determination of Tyrosine and Its Use in Determining the Tyrosine Content of Edestin, Casein, and Tobacco Mosaic Virus

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INTRODUCTION AND EXPERIMENTAL

Gerngross, Voss, and Herfeld (1) have reported that a purple or red color is obtained when a hot solution containing tyrosine and α -nitroso- β -naphthol is treated with concentrated nitric acid. Tests by these authors indicated that the reaction is specific for para-substituted phenols which are not substituted in the ortho-positions or have a methyl group in only one ortho-position. Quantitative determination of tyrosine by ordinary colorimetric methods was not accomplished by these workers because of the instability of the color. The excess nitric acid causes fading.

In the present method the acidity is increased by hydrochloric acid, and dilute nitric acid is used. When this is done the development and fading of the color takes place much more slowly and the fading can be postponed by cooling the solution at the proper time.

Diiodotyrosine is the only other amino acid known to be a constituent of proteins which gives a color with α -nitroso- β -naphthol and nitric acid. The method can therefore be applied directly to most protein hydrolyzates. It also seems to offer good possibilities for the determination of free phenol groups in proteins and protein derivatives.

Apparatus and Reagents

A Sheard and Sanford "Photometer" (2) was used for all determinations, with the green "Photometer" filter and a 1 cm. absorption cell.

A solution of α -nitroso- β -naphthol (Eastman) in 95% alcohol containing 120 mg. of the nitrosonaphthol per 100 cc. of solution. This solution has been kept in a refrigerator below 5°C. in an amber bottle for 2 months and at room temperature

in an amber bottle for one month with the same results as when it was freshly prepared.

A nitric acid solution prepared by diluting 12 cc. of concentrated nitric acid (sp. gr. 1.42) to 100 cc. with water.

Concentrated hydrochloric acid (sp. gr. 1.19).

Standard tyrosine solutions, containing from 0.08 to 0.30 mg. of tyrosine per 5 cc. The stock solution, from which these were prepared by diluting with water, contained 5 cc. of concentrated hydrochloric for each 100 mg. of tyrosine.

The Method

Five cc. of the tyrosine solution are placed in each of two 18 × 150 mm. Pyrex test tubes. One cc. of the α -nitroso- β -naphthol solution is added to one tube and 1 cc. of 95% alcohol to the other. To each tube is then added 2 cc. of the hydrochloric acid and 1 cc. of the nitric acid solution. The contents of the tubes are well mixed and the tubes shaken in a boiling water bath for exactly 47 seconds. They are then quickly placed in a water bath maintained between 20° and 30° and shaken there for one minute after which they are placed in the absorption cells and the reading made within 8 minutes of the removal from the cooling bath. No unnecessary time should elapse from the beginning of the addition of the reagents until after the solutions are cooled.

The solution containing the 95% alcohol is used in the "Photometer" as a reference solution instead of distilled water while making the reading of the red colored solution, i.e. the solution to which α -nitroso- β -naphthol has been added. This procedure eliminates errors due to the yellow color produced in the solution by heating tyrosine or the protein hydrolysis products with nitric acid.

Accuracy

An amino acid mixture was prepared. It contained 19 amino acids and ammonium sulfate and approximated an edestin hydrolyzate in composition. Quantities of the amino acids and ammonium sulfate to make a total of one gram were dissolved in water containing 2 cc. of concentrated hydrochloric acid and diluted to 100 cc. with water. Fifteen cc. of this solution were diluted to 100 cc. with water. Determinations on 4 cc. aliquots of the dilute solution, containing 0.240 mg. of tyrosine, gave 0.242 mg., 0.245 mg., 0.244 mg., and 0.242 mg. Determinations on aliquots containing 0.180 mg. gave 0.185 mg., 0.183 mg., 0.183 mg., and 0.185 mg. Determinations on aliquots containing 0.120 mg. gave 0.124 mg., 0.123 mg., 0.123 mg., and 0.124 mg.

Determination of Tyrosine in Proteins

Casein and edestin were dried in a vacuum desiccator over sulfuric acid for 48 hours. The tobacco mosaic virus was some of the same preparation used by Ross for amino acid determinations (3). It had been ultracentrifuged four times, frozen, and dried in vacuo. It was

dried to constant weight at 110°. Samples of all three proteins were weighed directly into acetylation flasks and hydrolyzed by reflux boiling with 20% hydrochloric acid. The samples of edestin and casein weighed between 450 and 1200 mg. The samples of tobacco mosaic virus weighed between 65 and 210 mg. In the case of edestin, 15 to 25 cc. of hydrochloric acid were used, with casein 15 to 20 cc., and with tobacco mosaic virus 5 to 10 cc.

The hydrolyzate was in each case transferred to a volumetric flask, made up to volume with water and filtered to remove insoluble humin. In making further dilutions, an aliquot of this stock solution was placed in a volumetric flask, neutralized with sodium hydroxide, then made slightly acid with hydrochloric acid and made to volume. It was found by trials with a tyrosine standard solution that the maximum quantity of sodium chloride left after neutralization and dilution of the hydrolyzates did not interfere with the determination.

The results of tyrosine determinations on the protein hydrolyzates are shown in Table I. Tyrosine added to protein hydrolyzates was

TABLE I

Determination of Tyrosine in Edestin, Casein, Tobacco Mosaic Virus

Protein	Hydrolysis sample	Tyrosine per cent	No. of determinations	Average deviation from the mean
Edestin	1	3.96	6	0.06
	2	4.05	6	0.12
	3	3.92	6	0.09
	Av. 3.98			
Casein	1	6.16	6	0.05
	2	6.08	6	0.11
	3	5.86	5	0.05
	Av. 6.03			
Tobacco Mosaic Virus	1	3.62	6	0.02
	2	3.54	6	0.04
	3	3.59	6	0.03
	Av. 3.58			

recovered quantitatively. For example, three determinations, each on 2 cc. of a diluted casein hydrolyzate plus 3 cc. of water, gave an average value of 0.100 mg. of tyrosine. Two determinations, each on 2 cc. of the same diluted hydrolyzate plus 3 cc. of a solution containing

0.04 mg. of tyrosine per cubic centimeter, gave identical values of 0.219 mg. of tyrosine.

DISCUSSION

Gerngross, Voss, and Herfeld (1) stated that the colored substance obtained in this reaction did not obey Beer's law. Absorption spectrum curves I and II, Fig. 1, obtained with two different concentra-

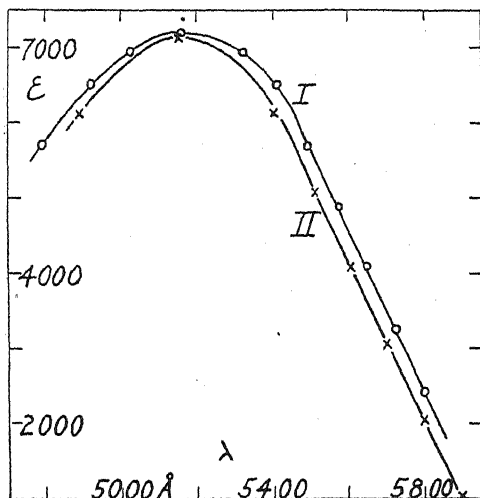


FIG. 1

Spectral Absorption Curves of Colored Solutions Produced by the Method Described Here, as Measured with a Hilger Spekker Photometer and a Tungsten Steel Spark Source

$$\epsilon = (1/cd) \log_{10} (I_0/I).$$

c = molar concentration of tyrosine.

d = length of absorbing solution in cm.

Curve I, 0.2 mg. tyrosine in 9 cc.

Curve II, 0.08 mg. tyrosine in 9 cc.

tions of tyrosine, show that Beer's law is obeyed in the zone of maximum absorption, between these two concentrations, but that the agreement is not good at other wave lengths.

Following the above procedure, but with heating periods from 40 to 55 seconds, the "Photometer" readings were almost constant. Between 25 and 40 seconds the readings were lower, due to a slight

turbidity, and with heating periods longer than 55 seconds there was a sharp increase in "Photometer" readings, *i.e.*, decrease in intensity of color. Tyrosine alone, the amino acid mixture described above, and protein hydrolyzates behaved alike in this respect. It is suggested that each operator run such a series and choose a heating period at the middle of the plateau in the curve, since the thickness of test tube walls, etc., may change somewhat the time required for the production of color.

When the reaction is carried out according to the procedure given above, the color remains stable for 8 to 10 minutes after removal from the cooling bath and then begins to fade gradually.

The methyl ester of dibenzenesulfonyltyrosine and thyroxin were found not to give the Gerngross-Voss-Herfeld reaction. The negative finding with thyroxin confirms the finding of these authors. 3,5-diiodotyrosine, the methyl ester of 3,5-diiodotyrosine, the methyl ester of tyrosine, the methyl ester of *N*-benzoyltyrosine, and the methyl ester of *N*-benzenesulfonyltyrosine, gave positive reactions. The diiodotyrosine and its methyl ester gave very much less color than tyrosine. In a quantitative test, the methyl ester of tyrosine assayed the same as tyrosine in equal molar quantities. A solution of tyramine hydrochloride assayed 30 per cent higher than a tyrosine solution of the same molar concentration. The positive reactions with diiodotyrosine and with its methyl ester are contrary to the rule, stated by Gerngross, *et al.*, regarding ortho-substitution compounds. This result seems to indicate that some of the iodine in these compounds is removed by the short heating period in a strong acid solution.

Contrary to the report by Gerngross, *et al.*, the author was unable to obtain a color with tyrosine, α -nitro- β -naphthol (Eastman) and nitric acid.

SUMMARY

A new method for tyrosine determination is reported. This method is based on the Gerngross-Voss-Herfeld color reaction, which is given by none of the amino acids contained in proteins except tyrosine and diiodotyrosine. The tyrosine content of edestin, casein and tobacco mosaic virus was found by this method to be 4.0, 6.0, and 3.6 per cent, respectively. It is suggested that this method seems to offer good possibilities for the determination of free phenol groups in proteins and protein derivatives.

The author is indebted to Dr. A. Frank Ross of the Department of Plant Biochemistry, University of Maine, formerly of the Rockefeller Institute for Medical Research (Princeton), for a purified sample of tobacco mosaic virus protein, and to Dr. Herbert E. Ungnade of the Chemistry Department, University of Missouri, for samples of the several tyrosine derivatives mentioned.

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Effect of Various Lighting Conditions on Riboflavin Solutions

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INTRODUCTION

The increased number of fluorometric determinations of riboflavin in food and biological fluids requested of this laboratory prompted a study of the conditions of illumination under which reliable determinations of riboflavin could be made without loss of riboflavin. The present study deals with the rate of destruction of pure riboflavin¹ exposed to direct daylight, diffused light and artificial light, as well as to rays in the visible and invisible spectra. Our investigations were limited to solutions of 8 μ g. of riboflavin per ml. in distilled water and in a mixture of *n*-butyl alcohol, pyridine, and acetic acid. Three series of assays were made in the aqueous medium, at pH 3.7, pH 6.3, and pH 9.0 respectively.

General Procedure

The solutions were placed in a 100 ml. pyrex beaker; the standard depth of solutions was 2.54 cm. Concentration change due to evaporation was negligible, and therefore no correction for this factor was necessary. A sample was taken at zero time, the concentration of which corresponded to the highest point on the standard curve. Additional samples were taken after 5, 15, 30, 60, 120 minutes of radiation. All of the samples for a given experiment were stored in the dark and measured fluorometrically as soon as the last sample had been withdrawn. A standard curve was run with each series of experiments, for there were fluctuations in the current and some

¹ Riboflavin "Roche," Crystalline Vitamin B₂. Hoffman-Laroche, Inc., Nutley, New Jersey.

instability of the apparatus itself. Standard solutions of *quinine* were used to confirm the reliability of the readings. The curves were established by plotting amounts of destruction of riboflavin in micrograms per 0.5 ml. against time.

Equipment and Preliminary Experiments

In the first series of experiments, aqueous solutions of riboflavin were exposed to various wave lengths of monochromatic light, using the Coleman Universal spectrophotometer model 11. The source of light in this apparatus is an 8.5 volt, 36 watt prefocussed coiled line filament lamp. The solution was placed in a square cuvette having an optical thickness of 13.06 mm. The determinations in monochromatic light were conducted at the following wave lengths—4100 Å for violet light, 5200 Å for green light, 6500 Å for red light.

The aqueous solution of riboflavin containing a trace of acetic acid (pH 3.7) was placed in the sample chamber of the spectrophotometer. The top opening of the chamber was closed by inserting the bakelite cover. At the aforementioned times 0.5 ml. aliquots were withdrawn. All fluorometric readings were made with the Klett fluorometer which is a two cell instrument. Throughout the experiment the null point method was employed.

For the *standard quinine solution* the primary filter was #5970, secondary #3060. Filter #5970 was used as permanent primary filter for both cells. The secondary filter used in checking the instrument with a solution of 0.25 mg. per liter of quinine was the thiochrome filter. The secondary filter used in conjunction with the riboflavin solutions was #3486. All filters were manufactured by Corning with the exception of the thiochrome filter which was furnished with the Klett fluorometer. In the fluorometer, the solutions were given a period of exposure of 15 seconds at the end of which time the readings were taken.

As a demonstration of the range of fluctuation, an entire set of readings obtained from a typical experiment is presented. The results obtained after exposure to red light at 6500 Å are given in Table I.

TABLE I

Riboflavin Solution after Exposure to Red Light at 6500 Å

Time in minutes	Readings	Values in micrograms per 0.5 ml.
0	181.0	4.00
5	177.3	3.95
15	184.0	4.07
30	184.5	4.08
60	182.0	4.01
120	182.0	4.01

A solution of 0.5 mg. quinine sulfate per liter was read at intervals against 0.25 mg. of quinine sulfate per liter in order to check on the stability of the fluorometer.

TABLE II
Light Source and Intensity

Nature of light	Natural light Intensity in Weston units	Artificial light (150 watt reflector)	
		Distance from light source	Intensity in Weston units
Direct sunlight	>1600	30 cm.	>1600
Daylight (in the shade)	400-500	50 cm.	800
Diffused daylight	4	100 cm.	300

These figures demonstrate that the variations of the readings were caused by fluctuations in the current and that no destruction occurred. Similar experiments were performed and repeated in violet and green light. In every case, after two hours' exposure, no destruction was observed. Difficulty was encountered in attempting to measure the intensity of light transmitted through the cuvette of the Coleman apparatus. However, it can be said with certainty that the light emitted in any case was of sufficient intensity to permit manipulations under conditions of fair visibility.

Obviously the illuminations of above mentioned intensities and qualities did not lower the fluorometric value of the riboflavin in two hours. Hence experiments were made in bright sunlight, daylight (shade), diffused daylight, artificial light, and ultra violet light. In these experiments the solutions were exposed to the light directly, without any intermediary glass screen.

Aqueous Solutions of Riboflavin

Exposure to Natural Light. The solution of riboflavin at pH 3.7 contained a trace of acetic acid. A phosphate buffer was used² for the pH 6.3 solution, and a borate buffer³ for the solution at pH 9. The light intensities are given in Weston Units,⁴ see Fig. 1.

² Composition of the Phosphate buffer solution:

Primary potassium phosphate (KH_2PO_4)	6.63 g.
Anhydrous secondary sodium phosphate (Na_2HPO_4)	2.56 g.
Distilled water to	1000.00 ml.

³ Composition of the borate buffer solution:

Solution I

Boric acid (BO_3H_3)	12.4 g.
Potassium chloride (KCl)	14.0 g.
Distilled water, to	1000.0 ml.

Solution II

Sodium hydroxide (NaOH)	8.0 g.
Distilled water to	1000.0 ml.
Buffer pH 9.0: 200 ml. of Solution I and 85.6 ml. of Solution II.	
Distilled water to	1000.00 ml.

⁴ A Weston "Master" Universal Exposure Meter model 715 was used. One Weston Unit is equivalent to one candle per square foot (brightness).

The riboflavin solution adjusted to pH 9.0 was made to contain 4 μ g. per 0.5 ml. However, the alkalinity alone brought this down at once to 1.5 μ g. per 0.5 ml. In order to determine whether we were dealing with a mere inhibition of fluorescence of the riboflavin at pH 9.0 or an actual destruction, the pH was readjusted to 4.0 but no recovery of riboflavin was detected.

From Fig. 1 we conclude that there is no detectable loss of riboflavin in 2 hours' exposure of aqueous solutions at a pH value of 3.7 to diffused daylight at an intensity of 2 to 6 Weston units.

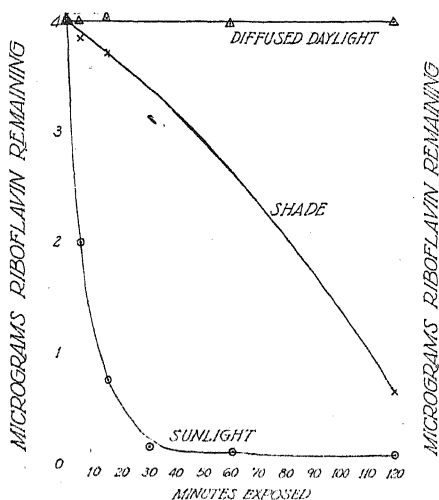


FIG. 1
Natural Light. pH 3.7

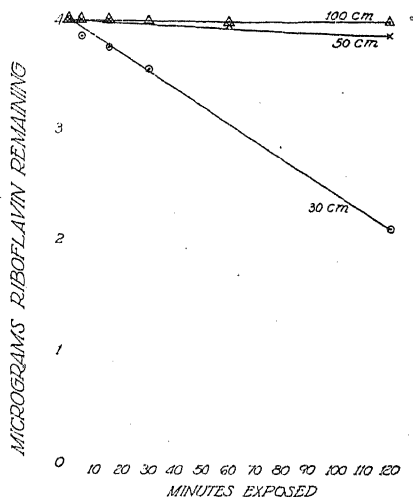


FIG. 2
Artificial Light. pH 3.7

Exposure to Artificial Light. A 150 watt reflector, type filament lamp was employed in this part of the study. The concentration of the solutions and their depth in the beaker were the same as before. The beakers were placed on a black or dark-green background. In order to obtain various light intensities by using only one source of light, the solutions were placed in rows, one above the other, at 30 cm., 50 cm., and 100 cm. from the source. The light was permitted to strike the solution surface perpendicularly with air as the only intermediate medium, see Fig. 2.

Exposure to Colored Lights: Red, Green, and Violet. The same 150 watt reflector was used in this experiment in conjunction with colored

cellophane filters.⁵ The absorption spectrum curves for these particular filters are given in Brode's "Chemical Spectroscopy" (3). The maximal transmission and percentage of light intensity transmitted were taken from these curves (Table III). The results are given in Figs. 3 and 4.

TABLE III
Characteristics of Colored Filters

Filter	Maximal transmission in millimicrons (Ref. (3), p. 269 and 444)	Percentage of light transmitted
Red	600	85
Green	500	46.8
Violet	420-440	75

It is obvious that the aqueous solutions at pH 6.3 are less resistant to the red, green, and violet light than those at pH 3.7. The expected

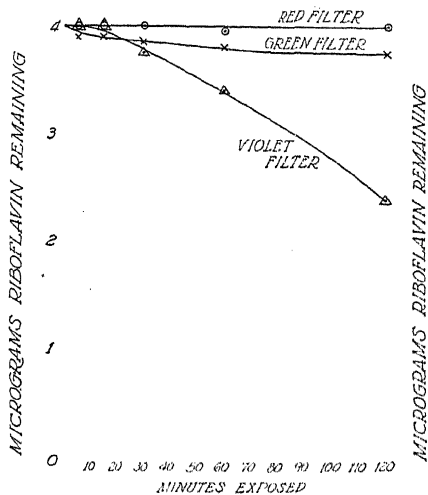


FIG. 3
Artificial Light
Distance 30 cm. pH 3.7

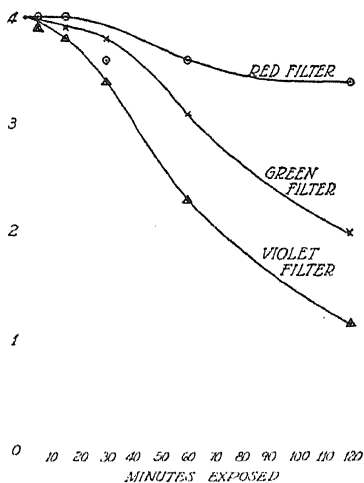


FIG. 4
Artificial Light
Distance 30 cm. pH 6.3

greater destruction of riboflavin by exposure to shorter wave lengths in the visible range is also confirmed.

⁵ Manufactured and supplied by the "Cellophane" Division of the E. I. duPont de Nemours & Co., Wilmington, Delaware.

Exposure to Ultra Violet Light. The apparatus used in performing these experiments was a Burdick quartz tube, mercury vapor lamp, type A-909, operated on 110 volts D.C. The lamp had been used for a total of approximately eighty hours. In these experiments, the test solutions were exposed directly to the source of radiation with the light striking the exposed surface perpendicularly, see Figs. 5 and 6.

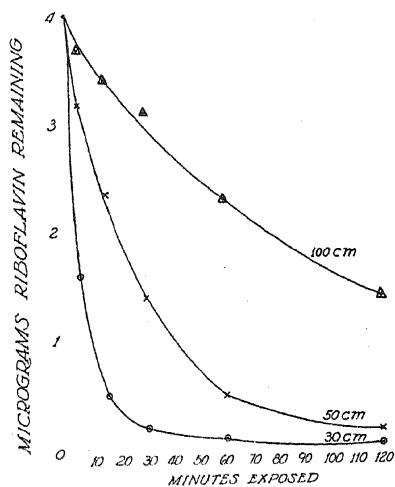


FIG. 5

Ultra Violet Light. pH 3.7

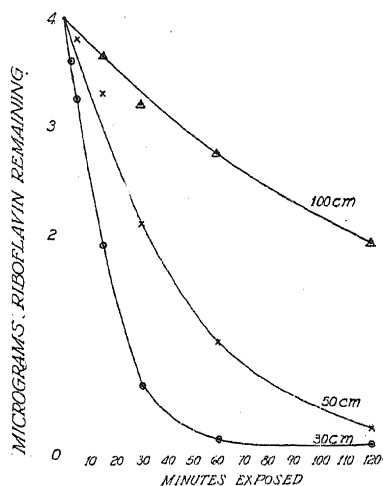


FIG. 6

Ultra Violet Light. pH 6.3

A systematic study at pH 9.0 was not made. The similarity exhibited by the curves obtained at pH 9.0 on single experiments indicate that the same pattern of disintegration was followed for distances up to 1 meter. At a distance of 30 cm. from the light source, practically complete destruction was attained after one hour's exposure for all pH ranges. When the distance was 50 cm., similar results were observed after two hours. It is worthwhile to note that exposure at 1 meter clearly shows the protective effect of an acid medium upon riboflavin. It may be surmized that the disintegration would still occur according to the same pattern if the distance were greater; however, no further evidence is available.

Irradiation of Solutions of Riboflavin in a n-Butyl Alcohol, Pyridine, Acetic Acid Mixture

Since organic solvents are used in the extraction of riboflavin from foods, etc., it was desirable to study the effect of the solvents on the light stability of riboflavin in these solvents. A mixture of 17 ml. butyl alcohol, 2 ml. pyridine and 1 ml. glacial acetic acid was used as the solvent in these studies.

A standard curve was run with each experiment. The experimental procedure, as far as exposure to light is concerned, was exactly the same as the one described for aqueous solutions. Blanks of this particular mixture were taken after exposure to the same quality of light, and the differences, if any, were taken into consideration in plotting the results. In general, greater irregularity was observed in the curves obtained from the *n*-butyl alcohol mixture than in those from the aqueous medium.

Throughout this series of experiments, a concentration of approximately 8-9 $\mu\text{g.}$ of riboflavin per ml. was used. However, since 0.5 ml. of solution was taken for sampling, our data will be given for that volume. Since previous experiments showed that the light intensity of the Coleman spectrophotometer was not sufficient to produce destruction, similar determinations with this apparatus were not performed.

n-Butyl alcohol mixture, 4.5 g./0.5 ml.

(a) *Sunlight.* Experiments with aqueous solutions of riboflavin in direct daylight were performed during the summer months. Since the butyl alcohol solutions could not be similarly treated until autumn, comparable results could not be obtained because of adverse weather conditions and the changed angle of incidence of the solar radiations.

(b) *Diffused daylight.* In this experiment only three samples were taken, the zero sample, the one-hour sample, and the two-hour sample. No destruction was observed. This intensity of 4 Weston units of light had no ill effect upon riboflavin, but it produced a perceptible eye fatigue after a prolonged period of time and hence is not the most desirable intensity of illumination for good working conditions.

(c) *Artificial light* (see Fig. 7). Contrary to expectations, this riboflavin solution in the organic solvent exhibited peculiar behavior when exposed to artificial light of different intensities. Discrepancies were observed between even identical experiments. In every case,

the loss of riboflavin appeared earlier during the exposure, but the final destruction amounted to slightly less than 60% of the original quantity of riboflavin exposed. After 60 minutes radiation, very little additional destruction was observed suggesting that some protective factor has been developed during the radiation or that some factor has been introduced which interferes with the accurate fluorometric estimation of riboflavin.

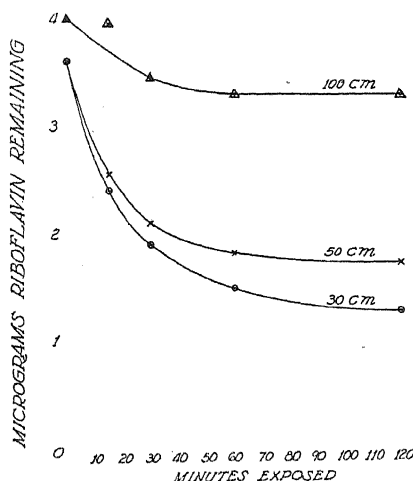


Fig. 7

Artificial Light
Butyl Alcohol Mixture

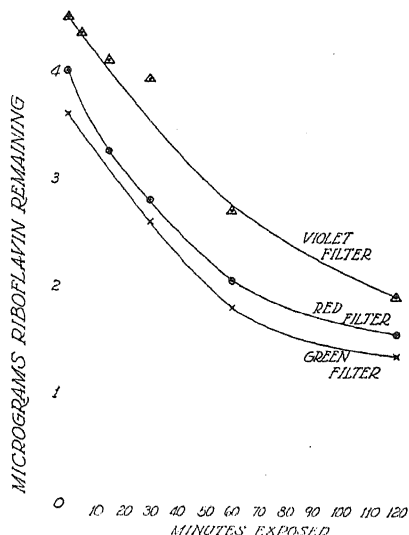


Fig. 8

Artificial Light
Butyl Alcohol Mixture

The radiation of these solutions by green, red, and violet light also causes different rates of destruction than in aqueous solutions. These rates do not differ very much in the organic solvent and the relative rates differ from those found for the aqueous solution (see Fig. 8).

Ultra Violet Light

This series of experiments was conducted as previously described. The curves—see Fig. 9—are not as regular as those obtained with aqueous solutions. This resulted in a repetition of what had already been observed when treating the mixture with artificial light under previous conditions. The butyl alcohol mixture which contains pyridine darkens after exposure to ultra violet light. The blanks taken before

and after the experiment showed an appreciable difference. Consequently, a slight error in the absolute and even relative results obtained was to be expected. The readings on the blanks for the butyl alcohol mixture exposed to ultra violet light were higher than readings obtained previous to exposure. This means that the final concentration is slightly less than shown on the graphs.

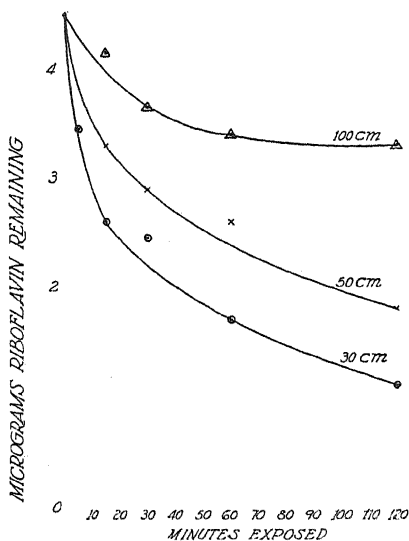


FIG. 9
Ultra Violet Light
Butyl Alcohol Mixture

The graphs showing the destructive effect of ultra-violet light on riboflavin at the aforementioned distances are similar in slopes to those of Fig. 7. The slope obtained for the 1 meter curve seems to indicate either that the protective effect of the mixture is considerable or that a protective factor has been formed or that this interferes with the accurate fluorometric estimation of riboflavin.

SUMMARY AND CONCLUSIONS

1. Aqueous solutions of riboflavin exposed to the light from the filament lamp of the Coleman spectrophotometer gave no evidence of destruction through the region of the spectrum ranging from the red to the violet.

2. Although the destructive effect of strong daylight on riboflavin is well known, this systematic study proves that exposure to direct sunlight even for a brief period of time is sufficient to cause considerable destruction.

3. Diffused daylight having an intensity not higher than 6 Weston units does not harm the substance. Hence the manipulations necessary for the extraction of riboflavin from foods or biological fluids can be made safely at this light value.

4. In artificial light, aqueous solutions of riboflavin between pH 3 and 4 are quite stable at a minimum distance of 1 meter from the light source for a period of 2 hours. If daylight is excluded, quantitative riboflavin determinations at this pH can be carried on under ordinary lighting conditions with little if any loss.

Near neutrality, riboflavin solutions exhibit a slight instability, at 1 meter from the 150 watt lamp used. This instability would be imperceptible under the conditions described for solutions at pH 3 to 4.

Solutions of riboflavin in a mixture of *n*-butyl alcohol + pyridine + acetic acid are relatively unstable for precise determinations under the conditions imposed by this experiment. Consequently, quantitative determinations should be carried on at a maximum light intensity of 6 Weston units.

5. Ultraviolet light, as expected, destroys riboflavin very rapidly. For any given period of exposure, ultra violet light produced greater destruction than any of the other wave lengths used.

6. A 150 watt reflector screened with a red cellophane filter does not affect adversely aqueous acid riboflavin solutions and provides adequate illumination for laboratory operations.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to the Officers of the Elgin State Hospital as well as to the Committee on the Nutritional Aspects of Ageing (National Research Council) for having rendered this work possible.

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Inhibition of the Succinoxidase System by Cysteine and Cystine*

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INTRODUCTION

The effect of cysteine and cystine on succinic dehydrogenase was first studied by Hopkins and co-workers (1), using a system involving methylene blue as a hydrogen acceptor. They incubated minced and washed pig's heart with cystine for four hours, then washed the preparation, and showed a loss in the activity which could be completely restored by similar treatment with cysteine. From this and similar experiments with glutathione (1, 2), the conclusion was drawn that the activity of succinic dehydrogenase involved an $-SH$ group; the reduced form being active; the oxidized, inactive.

The effect of many inhibitors on succinic dehydrogenase was studied by Potter and DuBois (3), who used tissue homogenates to determine the presence and role of $-SH$ groups in this enzyme. They indicated that succinic dehydrogenase contained an active sulfhydryl group which was located between two carboxyl affinity points. The further conclusion was drawn that the enzyme functioned by oscillating between the thiol ($EnSH$) and the oxidized free radical ($EnS\cdot$) forms, rather than the thiol-disulfide equilibrium proposed by Hopkins (2). Potter and DuBois discussed the inactivation caused by cysteine and cystine from this standpoint and reported that both these compounds inhibited the enzyme when they were added to the final reaction mixture which contained cytochrome *c*, in the presence of which sulfhydryl is readily oxidized.

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In this investigation the action of cystine and cysteine on the succinoxidase system is studied in greater detail. Methods of determining the extent of inhibition described by previous investigators were found to be nonreproducible unless certain additional variables were controlled. It is shown in this paper that, in order to obtain reproducible results in duplicate experiments, it is necessary to maintain a constant time of incubation of the tissue homogenate before the inhibitor is added, in addition to controlling the concentration of inhibitor and its time of contact with the enzyme. The degree of inhibition observed has been shown to be dependent on the concentration of four-carbon dicarboxylic acids present. Therefore, inhibition values obtained for undialyzed crude preparations have little or no quantitative significance if applied directly to the enzymes concerned.

METHODS

White mice of an inbred Swiss strain were obtained from Dr. Harry Waisman. After weaning they were maintained on stock ration¹ and water *ad libitum* plus occasional greens.

A tissue homogenate was prepared by decapitating the mice and allowing the blood to drain for a short time. The tissue was extirpated, rinsed with distilled water, and immediately packed in finely cracked ice. After cooling, bits of fat and connective tissue were removed, the tissue was blotted between moistened filter papers, and rapidly weighed. A homogenate of the tissue was prepared by the technique of Potter and Elvehjem (4) in ice-cold redistilled water using a pre-chilled tube and pestle. This homogenate was immediately pipetted into the reaction flasks to which all other reactants had been previously added.

A conventional Warburg apparatus at 37°C. was used in all experimental and analytical work. The pH was determined in all cases with a Beckman pH meter (glass electrode).

The activity of succinic dehydrogenase was determined by the method of Potter (5) as modified by Schneider and Potter (6). A summary of the components of the final reaction mixture is as follows: 0.4 ml. of 0.25 *M* phosphate buffer (pH 7.4), 0.1 ml. of 4×10^{-4} *M* cytochrome c, 0.3 ml. of 0.5 *M* sodium succinate (pH 7.4), 0.1 ml. of 0.012 *M* CaCl_2 , 0.1 ml. of 0.012 *M* AlCl_3 , the desired amount of homogenate, solution of inhibitor, and redistilled water to make 3.0 ml. The gas phase was air and 0.2 ml. of 10% KOH and a small strip of filter paper were placed in the center well to absorb CO_2 . The pH of this final reaction mixture was 7.4 as determined electrometrically.

Eastman Kodak Company cysteine hydrochloride was used. Its purity was determined by measuring the oxygen uptake on catalytic oxidation by cupric ions. By this means the cysteine was shown to be approximately 96% pure. Solutions were freshly

¹ B-B Laboratory Rabbit Diet, Maritime Milling Company, Inc., Buffalo, New York.

prepared in redistilled water and neutralized to pH 7.4 just before adding to the reaction flask.

EXPERIMENTAL

The inhibition of the succinoxidase system in a tissue homogenate by $M/500$ cysteine was reported by Potter and DuBois (3) to be about 93%. On repeating their work markedly lower values were obtained, and these values showed great individual variation, ranging from 40 to 55% inhibition. In a series of experiments in which previously reported variables were held constant and other possible factors altered, the effect of an additional source of variation on the degree of inhibition was determined. In this manner the data in Fig. 1 were

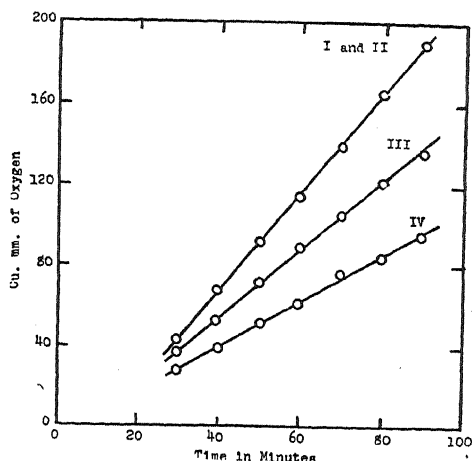


Fig. 1

Effect of Cysteine on the Activity of the Succinoxidase System, With and Without Prior Incubation of the Homogenate

Test system as described in text. Two-tenths ml. of a 2% homogenate of mouse kidney added. Cysteine concentration of $M/500$. Time of succinate addition was zero time.

Curve I, control—no inhibitor added; succinate added 20 minutes after homogenate.

Curve II, control—no inhibitor added; succinate added 60 minutes after homogenate (identical with Curve I).

Curve III, cysteine present when homogenate added; succinate added 20 minutes later.

Curve IV, cysteine added after 40 minutes incubation of the reactants and homogenate; succinate added 20 minutes later (60 minutes after addition of homogenate).

obtained, which clearly showed the marked influence of the length of time of incubation of the homogenate with calcium ions before the inhibitor was added, or the *incubation time*, on the degree of inhibition of the succinic dehydrogenase activity.

A more complete study of the effect of the incubation time followed. By varying only the length of the incubation time, the data plotted in Fig. 2 were obtained for $M/500$ cysteine, with a contact time (*vide*

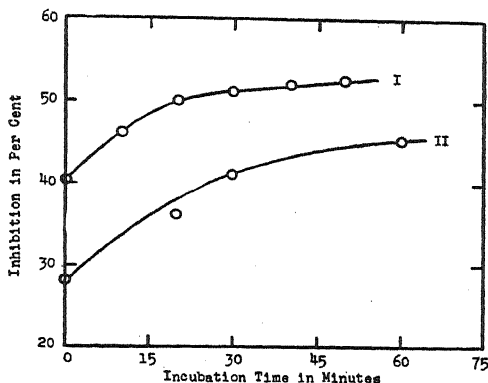


FIG. 2

Effect of Incubation Time on the Degree of Inhibition

Length of time between addition of homogenate and addition of cysteine was termed the incubation time. Test system as described in text. $M/500$ cysteine in the reaction flasks. Two-tenths ml. of a 2% homogenate of mouse kidney added.

Curve I, cysteine added at times indicated; succinate added 30 minutes later.

Curve II, cysteine added at times indicated; succinate added 20 minutes later.

infra) of 30 minutes for Curve I and of 20 minutes for Curve II. The degree of inhibition was found to be a direct function of the incubation time, approaching a nearly constant value after 30 to 40 minutes. Since the change in inhibition with a change in incubation time was very small at about 40 minutes, this time was selected as a suitable incubation time for subsequent experiments. By maintaining a constant incubation time, uniform results were obtained in duplicate experiments, even when on the same tissue from different animals.

Since a reproducible method for measuring the inhibition had been established, the effect of other variables could be determined. A series of measurements were made of the degree of inhibition *vs.* the time of contact of the cysteine-cystine system with the enzyme before the suc-

cinatc was added, or the *contact time*, with all other variables constant. Change in the contact time produced a markedly altered degree of inhibition (see Fig. 3). The degree of inhibition was found to increase rapidly as the contact time was increased. When succinate and cysteine were added together, the enzyme was still inhibited to the extent of 15 to 20%.

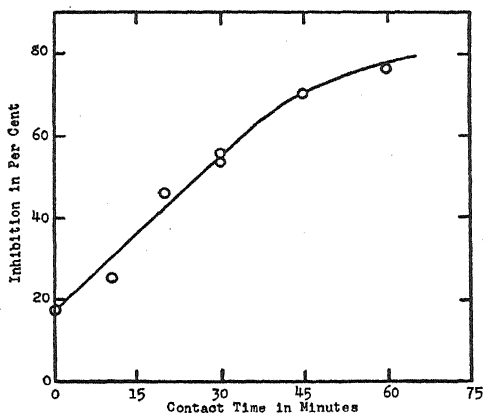


FIG. 3

Effect of Contact Time on the Degree of Inhibition

Length of time between addition of the inhibitor and addition of the substrate was termed the contact time. Test system as described in text. $M/500$ cysteine in the reaction flasks. Two-tenths ml. of a 2% homogenate of mouse kidney added. Incubation time of 40 minutes.

In order to ascertain the effect of the concentration of cysteine added at the beginning of the contact period on the degree of inhibition, it was necessary to select a constant incubation time and a constant contact time. An incubation time of 40 minutes was selected for these experiments for reasons previously discussed. A contact time of 30 minutes was arbitrarily selected since it was sufficiently long to insure complete equilibrium of the reactants and still not long enough to introduce a serious error due to normal inactivation of the enzyme. Under these conditions the results plotted in Fig. 4 were obtained. The degree of inhibition varied directly as the concentration of the inhibiting agent, the curves approaching the concentration axis as an asymptote. These results showed that under these conditions a con-

centration of 10^{-5} or even 10^{-6} *M* cysteine in the reaction vessel at the beginning of the contact period would cause an appreciable inhibition of the enzyme.

The effect of the incubation time on the degree of inhibition was a factor which had not been considered by previous investigators. As shown in Fig. 2, an increase in the incubation time resulted in an increased inhibition. This would tend to indicate that a substance was present in the homogenate which prevented the inhibitor from exerting

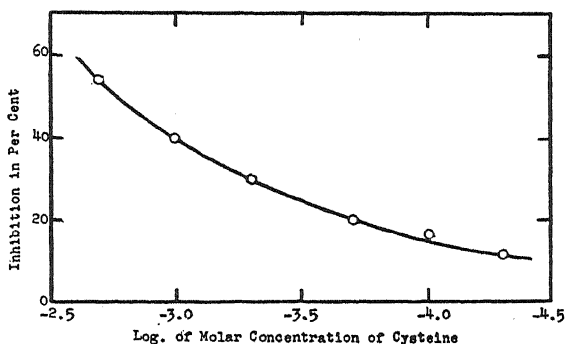


FIG. 4

Effect of Cysteine Concentration on the Degree of Inhibition

Test system as described in text. Two-tenths ml. of a 2% homogenate of mouse kidney added. Incubation time of 40 minutes. Contact time of 30 minutes.

its normal effect and that, on incubation, this substance was no longer active. Furthermore, the rate of destruction of this hypothetical substance decreased with time and had ceased entirely at about 30 to 40 minutes.

If this decrease in the rate of destruction of the hypothetical substance with increased incubation time were attributed ultimately to an acceleration in the destruction of cozymase by calcium ions as elucidated by Swingle, Axelrod, and Elvehjem (7), the following hypothesis fits the observed data. The hypothetical substance which prevents the inhibiting agent from exerting its normal effect is an acid or acids of structure similar to succinic acid, *i.e.*, probably a four-carbon dicarboxylic acid. Following the theory developed by Potter and DuBois (3), such a substance would partially saturate the carboxyl affinity points of the enzyme, thus protecting the centrally located

sulfhydryl group. By this means the enzyme would remain in the active reduced state since the inhibitor could not react to form its inactive complex. In addition, the four-carbon dicarboxylic acid must have a pathway of destruction through a cozymase-linked system, and this in turn would be destroyed by incubation with calcium ions.

Fumaric and malic acids are four-carbon dicarboxylic acids which have the above characteristics. Their conversion to oxalacetic acid is by a cozymase-linked mechanism, the oxalacetic acid formed then being decarboxylated by other systems. The conclusion that malic and fumaric acids are so concerned in the inhibition of the succinoxidase system by the cysteine-cystine system has been verified by the following experiments.

If the postulated hypothesis is correct, the addition of malate and fumarate to the reaction vessel should protect the enzyme from the specific action of the cysteine-cystine system. This should occur with very small amounts of these substances since their concentration in the homogenate would be extremely low. Furthermore since the enzyme, fumarase, is present in the tissue homogenate, fumarate and malate should show a very similar ability in protecting the enzyme against the action of the inhibitor. The data obtained by adding fumarate and malate to the reaction flask before adding the inhibitor are plotted in Fig. 5. The deviations of the individual points from the smooth curve were small considering that three different animals were used in obtaining the data. Malate and fumarate exerted a very similar action in protecting the enzyme against inhibition, and were effective in extremely low concentrations. In the lower concentrations, the curve obtained by plotting inhibition against concentration was asymptotic to the normal inhibition. This showed the effect of added fumarate and malate to be strictly additive to the hypothetical substance present in the tissue homogenate. These results indicated that the addition of four-carbon dicarboxylic acids would prevent the inhibition of succinic dehydrogenase by the cysteine-cystine system.

That the effect of incubation was actually due to the destruction of a cozymase-linked system is shown by the data in Fig. 6. A sample of the homogenate was divided into two portions: to one a solution of cozymase² was added; and to the other, redistilled water. Both were incubated at 37°C. for 30 minutes and, after adding to the reaction

² We are indebted to Merck and Company, Inc., Rahway, New Jersey, for a generous gift of cozymase.

vessels, the degree of inhibition was determined in the usual manner. The results showed that incubating a sample of homogenate at 37°C. with added coenzyme I increased the inhibition of succinic dehydrogenase by about 20% over a similarly treated control with no coenzyme I added. The addition of cozymase caused the cozymase-linked system to react faster, thus removing a greater portion of the available substrate. Since the presence of this substrate protected the enzyme from the inhibiting agent, its removal increased the degree of inhibition as measured by the activity of the succinoxidase system.

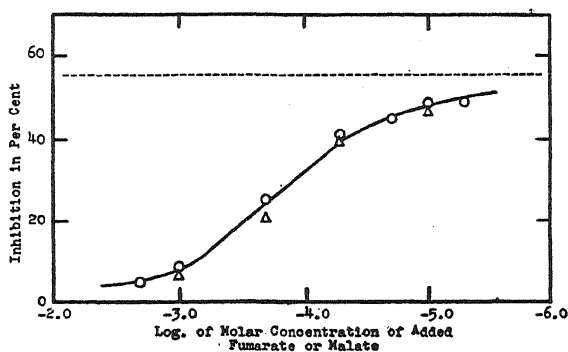


FIG. 5

Effect of the Presence of Fumarate or Malate on the Degree of Inhibition

Test system as described in text. Cysteine concentration of $M/500$. Fumarate or malate added to reaction flask before homogenate. Two-tenths ml. of a 2% homogenate of mouse kidney added. Incubation time of 40 minutes. Contact time of 30 minutes.

Experimental points are: \circ , fumarate; \triangle , malate; —, normal inhibition with no added fumarate or malate.

It was now evident that fumarate and malate satisfied the requirements imposed by the hypothesis. In addition, the possible action of oxalacetate and succinate as those most likely to similarly influence this system remained to be considered. Succinate in any appreciable concentration might be eliminated since there was no measurable oxygen uptake before the substrate itself was added. The presence of any oxalacetate was minimized because this compound acts as a specific inhibitor of succinic dehydrogenase and would therefore inhibit the controls as well. It is possible that both these substances and other

compounds of similar nature might be present in the homogenate, but only in extremely small concentrations.

With the experiments of Hopkins and co-workers (1, 2) in mind, the nature of the cysteine-cystine system was considered in some detail to see whether the cysteine or some oxidation product was inhibiting

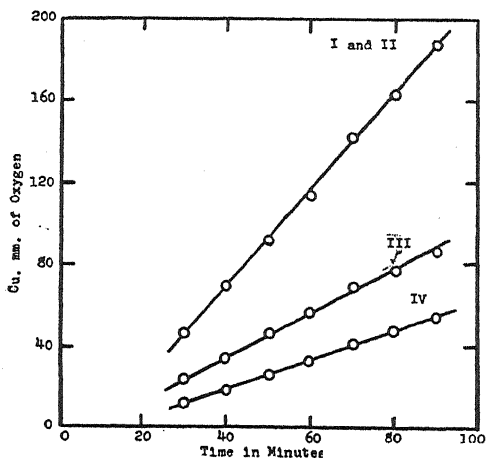


FIG. 6

Influence of Added Cozymase on the Degree of Inhibition

Test system as described in text. Cysteine concentration of $M/500$. Two-tenths ml. of a 2% homogenate of mouse kidney added in all cases. Incubation time of 40 minutes. Contact time of 30 minutes. Addition of succinate at zero time.

Curve I, control—no inhibitor added; 2% homogenate incubated at 37°C. for 30 minutes with no cozymase added before adding 0.2 ml. to the reaction flask.

Curve II, control—no inhibitor added; 2% homogenate similarly incubated but with 50 μ g. of cozymase per ml. added (identical with Curve I).

Curve III, inhibitor present; 2% homogenate incubated as above and with no cozymase added.

Curve IV, inhibitor present; 2% homogenate incubated as above but with 50 μ g. per ml. of cozymase added.

the enzyme. It was noted that in the reaction mixture, the addition of cysteine immediately produced a rapid oxygen uptake, but the rate of uptake soon decreased and in twenty minutes had ceased entirely. In all these experiments this rapid initial reaction was allowed to complete itself before readings of the oxygen uptake were attributed to the action of succinic dehydrogenase. Both cytochrome *c* and

cytochrome oxidase, added as tissue homogenate, were necessary components of the system which oxidized the cysteine. The experiments listed in Table I show that cysteine in the reduced form did not inhibit

TABLE I
Degree of Inhibition Due to Unoxidized Cysteine

Experiment	Description	Inhibition percent
1	Normal inhibition; 40 minute incubation period; 30 minute contact time	55
2	Normal inhibition; 40 minute incubation time; zero contact time; (cysteine added with succinate)	17
3	Cytochrome <i>c</i> absent until end of contact time; incubation time of 40 minutes; contact time of 30 minutes; (succinate and cytochrome <i>c</i> added together)	19

Normal inhibition implies that cytochrome *c* was present in the reaction mixture from the start. Test system was described in text with modifications noted. Cysteine concentration of $M/500$. Two-tenths ml. of a 2% homogenate of mouse kidney added.

the enzyme. By eliminating cytochrome *c* from the reaction mixture until the end of the contact period, the influence of the reduced form of the inhibiting substance could be determined. Cysteine, itself, proved to have no effect, and the degree of inhibition was the same as that obtained when the cysteine was added with the succinate, *i.e.*, a zero contact period. It was evident, therefore, that cysteine as such had no effect on the enzyme but, after being oxidized, exerted a specific inhibitory effect. This oxidized form is in all probability the dimer, but this has not yet been demonstrated conclusively.

In order to make better comparison between these results and those obtained previously by Potter and DuBois (3), different tissues were studied since the work of these investigators was done with homogenates of rat liver; and the present work, with mouse kidney. Under identical conditions,³ homogenates of mouse kidney and rat liver gave inhibition values of 55 and 49% respectively. These data indicated that there was no great difference in the degree of inhibition secured with homogenates of several different tissues when under the same

³ Test system as described in text. Cysteine concentration of $M/500$. Incubation time of 40 minutes. Contact time of 30 minutes. Homogenates: 0.2 ml. of a 2% homogenate of mouse kidney; 0.15 ml. of a 4% homogenate of rat liver.

conditions. The striking difference between the inhibition obtained in this study and that reported by Potter and DuBois can be explained on the basis of the hypothesis previously outlined. Potter (personal communication) used a homogenate that had stood at least four hours in the cold. During this time the cozymase-linked malic dehydrogenase had probably catalyzed the decomposition of malate in the homogenate, thus reducing the concentration of malate, fumarate, and similar substances to a point where only about 10 per cent of the enzyme was protected from inactivation by the cysteine-cystine system.

DISCUSSION

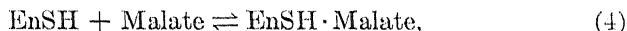
It is now possible to surmise to a considerable extent the reactions involved in determining the extent of inhibition. Succinic dehydrogenase must exist in an equilibrium,



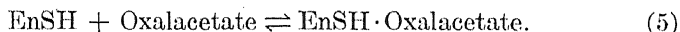
where *EnSH* represents the reduced enzyme; *EnS*·, the free radical oxidized form; and *EnSSEn*, the oxidized dimer. Hopkins and co-workers have shown that only the reduced enzyme is active, so we must have a series of equilibria,



and



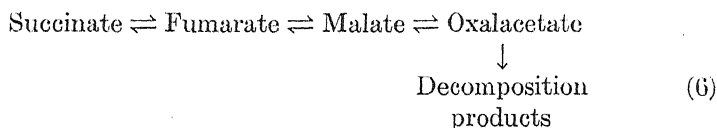
where the right member of each equation represents the enzyme substrate complex. Since we are dealing with succinic dehydrogenase, the equilibrium constant, *K*, of Eq. 2 must be much greater than those of Eq. 3 and Eq. 4, but the *K*'s of the latter two equations must be of appreciable magnitude since the bulk of the enzyme is protected from inhibition when either malate or fumarate is present. In addition to these three reactions, the enzyme will react with any oxalacetate present.



Indications are that *K* of Eq. 5 is much greater than for Eq. 3 and Eq. 4 since oxalacetate is a strong and specific inhibitor of succinic dehy-

drogenase (7). Since the above equations refer specifically to succinic dehydrogenase, the enzyme-succinate complex of Eq. 2 is dehydrogenated to the fumarate complex, but no further reaction is implied for the complexes formed in Eqs. 3, 4, and 5.

In a homogenate where the coenzymes are diluted but still active, the several four-carbon dicarboxylic acids are in equilibrium,



where malic dehydrogenase is the only cozymase-linked enzyme to be considered in this study. The precursors of succinate may tend to increase its concentration, but in the presence of an active succinic dehydrogenase they will have little effect.

The result of the addition of calcium ions to this system has been previously shown by Swingle, Axelrod, and Elvehjem (7). The effect is to accelerate the destruction of cozymase by activating the cozymase nucleotidase and thus prevent the formation of oxalacetate. This prevents further destruction of fumarate and malate after about 40 minutes incubation under the conditions of this investigation.

On the addition of cysteine to a system containing cytochrome *c* and tissue (implying cytochrome oxidase), an immediate oxidation takes place and may result in an equilibrium,



where *CySH* represents cysteine; *CyS*·, the free radical oxidized form; and *CySSCy*, cystine, the oxidized dimer. In the previous summary of the reaction, the conclusion was finally indicated that the equilibrium was far in the direction of the oxidized form, and in this discussion we need consider only the free radical and its dimer. The inhibition reaction itself may be represented by two different reactions,



or



where Eq. 8 is that proposed by Potter and DuBois (3); and Eq. 9, by

Hopkins and co-workers (1, 2). Eq. 8 implies that $EnS\cdot$, the free radical oxidized enzyme, is present at equilibrium and reacts with $CyS\cdot$, the free radical oxidized cysteine, to form a complex containing both enzyme and cysteine residues. Eq. 9, on the other hand, implies a selective oxidation of the enzyme by the cystine to form a dimer of the oxidized enzyme. Present data cannot determine which of these two mechanisms is correct, but that of Potter and DuBois corresponds more closely to current views. Regardless of the mechanism the complex formed is very stable, and there are no indications of reversibility of the inhibition reaction. After a given amount of inhibition has occurred and succinate is added, the oxygen uptake is a strictly linear function with time. There is no indication of an increase in the rate of oxygen uptake and, therefore, the amount of enzyme present may be assumed to be constant with time. As a result Eq. 8 and Eq. 9, whichever is correct, are written as irreversible reactions.

The equilibrium involving the enzyme and its several complexes (Eqs. 2, 3, and 4) must be entirely reversible. This is shown by the fact that the degree of inhibition is a function of time. The inhibition reaction serves to lower the concentration of uncombined active enzyme, resulting in a further decomposition of the enzyme-complex. If we were to assume the inhibition reaction to be a rapid one, the curve of inhibition *vs.* time would be determined by the ease of decomposition of the enzyme-complex. In the homogenate, the concentration of substances which will form an enzyme-complex will effectively determine the extent of inhibition in a given time.

The addition of malate and fumarate will serve to increase the amount of enzyme in complex form and lessen the amount of free active enzyme. This will effectively serve to decrease the extent of inhibition in a given time. Similarly incubation of the homogenate with coenzyme I, by lowering the concentration of fumarate and malate according to the mechanism previously discussed, will serve to increase the extent of inhibition by maintaining a greater concentration of uncombined enzyme at equilibrium conditions.

By similar reasoning, incubation with calcium ions will stop the decomposition of malate by accelerating the destruction of coenzyme I since the decomposition of malate is a cozymase-linked system. This will maintain a constant amount of fumarate and malate in the homogenate and make reproducible inhibitions under similar conditions possible.

The results plotted in Fig. 5 introduce a microanalytical method for determining the amount of these four-carbon dicarboxylic acids in a tissue sample. Since the inhibition is a function of the amount of these substances present in the flask, the straight line obtained with added fumarate and malate can be extrapolated to any degree of inhibition found. It is beyond the scope of this paper to report on a detailed investigation, but preliminary work indicates that although large errors are involved, a fair approximation of the concentration of the four-carbon dicarboxylic acids can be made.

In the preceding discussion, an attempt is made to apply the results of this study to the reactions and equilibria involved in the system under consideration. The use of tissue homogenates has been an essential element in this investigation, for with purified preparations the effects on which these conclusions are based would not have been apparent. In studies of this type, where one is interested in obtaining information relating to physiological processes, the value of homogenates over other types of preparations is again demonstrated.

As a result of this investigation, inhibition values recorded by previous investigators, where homogenates or tissue minces were allowed to stand an indefinite time before adding the inhibiting substance, are shown to be nonreproducible. In order to obtain reproducible results not only the concentration and contact time of the inhibiting agent but also the age and incubation time of the homogenate must be carefully controlled.

The values for the degree of inhibition and the resulting curves obtained in this study are applicable solely for a definite tissue preparation and under the specific conditions noted. Conclusions drawn from these and similar results as to the nature of the enzyme molecule itself are thus of doubtful validity. In order to obtain reliable inhibition values dependent on the enzyme itself, either dialyzed homogenates or purified enzyme preparations must be employed.

SUMMARY

1. The extent of inhibition of the succinoxidase system obtained with cysteine solutions has been shown to depend on the age and on the time of incubation of the tissue homogenate before the inhibitor is added.
2. The prior addition of very small concentrations of malate and fumarate has been shown to protect the enzyme against the action of the inhibitor.

3. The incubation of the homogenate with a solution of cozymase before adding the inhibitor serves to increase the extent of inhibition.

4. The effect of incubation of the homogenate with calcium ions is to reduce the change of inhibition values on further incubation and thus to obtain reproducible values of the inhibition.

5. All this evidence is in support of the hypothesis that the extent of inhibition is dependent on the concentration of four-carbon-dicarboxylic-acids, specifically fumarate and malate, in the homogenate. These compounds are destroyed on incubation through a cozymase-linked enzyme system, which is itself destroyed on incubation with calcium ions. Therefore incubation with calcium ions results in a constant concentration of malate and fumarate, and reproducible inhibition values can be obtained.

6. The above hypothesis has been used to speculate further on the reactions and equilibria involved in this complex enzyme mechanism.

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Protein Intake and Pyridoxin Deficiency in the Rat ¹

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Recent studies in this laboratory (1) revealed that the capacity of the rat to store limited quantities of pyridoxin may lengthen or shorten the time required for the appearance of the skin lesions on pyridoxin deficient diets, depending on the previous nutritional history of the animal. In this paper we present evidence showing that another factor, the protein level of the diet, also influences the time of appearance and the severity of the acrodynia.

In our previous studies, we noticed that the time of appearance of the skin lesions in rats receiving 18 and 20% casein diets which was reported in the literature to be 6 to 8 weeks, differed appreciably from that observed in our animals on a 30% casein diet, which showed the symptoms in about 30–35 days. Moreover, in our experiments, supplements of corn oil given to rats on a 30% casein diet were without effect on the dermatitis whereas numerous cures have been reported when corn oil was fed with an 18 or 20% casein diet. György and Eckardt's observations (2) on the effect of egg white on pyridoxin deficient rats suggested to us the possibility of a relationship between pyridoxin and protein metabolism. In an attempt to provide a more adequate diet to their animals, these workers fed 10% of heated egg white in addition to the usual 18% casein. This addition increased the total protein content of the diet by about 10%. On the ration without egg white, 70.7% of the rats developed acrodynia whereas 87.4% of those receiving the egg white showed the symptoms. No supplements other than thiamin and riboflavin were given to the rats.

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EXPERIMENTAL

Our experiments were carried out in three parts at different times, the first two during the spring and summer of 1941 (Experiments 1 and 2) and the third during the summer of 1943 (Experiment 3). In all cases, weanling albino rats of the Wistar strain reared in our laboratory were employed. The basal diet had the following percentage composition: Casein (Labco), 30; sucrose, 55; Crisco, 5; cod liver oil (U.S.P., Squibb), 3; Osborne and Mendel salts, 7. The protein content of this diet was varied at the expense of sucrose. The composition of the rations is given in Table I. The food intake was measured in Experiments 2 and 3. The animals were weighed regularly and observed daily for symptoms of acrodynia.

The results of our experiments are summarized in Table I. In all cases skin lesions appeared first in the animals receiving the higher levels of protein, and were delayed or absent in those receiving the lowest level. Also, 90% of the rats receiving the 30, 45, and 60% casein diets developed symptoms as compared with 60% of those on the 15% casein diet. Furthermore, the acrodynia that developed on the low protein diet was a very mild form in most cases, contrasting sharply with the severe symptoms exhibited by the animals on the higher protein levels. The skin lesions appeared first on the paws and the tip of the snout and then spread until the lower limbs and the head became involved. In advanced cases the eyes appeared to be closed by a sticky exudate. Frequently "spectacle eye," swelling of the tail, thickening of the ears, and hematuria were observed, especially in animals which did not succumb early to the deficiency.

As shown in Table I, the food intake was approximately the same at the various protein levels. The data also reveal that the growth of the animals differed little among the various groups. This point is important, since a more rapid growth rate at the higher protein levels might be interpreted as an equally possible cause of early and severe dermatitis. Therefore, the conclusion is justified that the protein intake was the main controlling factor of the gross symptoms observed.

DISCUSSION

Our findings suggest a metabolic relationship between pyridoxin and protein. This idea has been advanced by other workers from other evidence. McHenry and Gavin (3) have observed that in rats fed a high protein diet, a synthesis of fat from protein is evident only when pyridoxin is included in the diet. Lepkovsky, *et al.* (4) have isolated xanthurenic acid from the urine of pyridoxin deficient rats, and have shown that this substance, suspected as an intermediate in tryptophan metabolism, actually originates in the dietary tryptophan. They suggest, on the basis of a preliminary report of the present investigation (5), that the aggravating effects of high protein diets on pyridoxin deficient rats may be due to the larger amounts of tryptophan ingested. This idea is not supported by preliminary studies which we have carried out in this laboratory (6). In this connection it is of interest

TABLE I

Data Showing the Effect of the Level of Protein Intake upon the Resistance of Rats to Pyridoxin Deficiency

	Experiment 1 ^a			Experiment 2 ^a				Experiment 3 ^b		
	(a) ¹	(b) ²	(c) ³	(a) ⁴	(b) ⁴	(c) ⁴	(d)	(a) ⁵	(b)	(c) ⁶
Amount of casein in the diet (%)	15	30	45	15	30	45	60	15	30	45
No. of rats	5	2	5	5	5	5	5	8	8	9
Growth in g./day at different times:										
10th day	1.0	1.0	0.8	1.1	1.7	1.2	0.8	1.0	1.3	1.1
30th day	0.6	0.4	0.5	0.7	0.9	0.7	0.2	0.8	0.6	0.7
50th day	0.4	0.1	0.2	0.6	0.6	0.3	—	0.7	0.5	0.7
Food intake in g./day at different times:										
10th day	—	—	—	4.2	4.1	3.6	4.0	4.7	4.5	4.2
30th day	—	—	—	3.7	4.4	3.5	3.0	4.6	4.3	4.1
50th day	—	—	—	3.7	4.4	3.3	—	4.5	4.3	4.5
Time of appearance in days of various stages of the acro-dynia and (in parentheses) number of animals affected										
First symptoms	62(1)	30(2)	26(4)	60(3)	39(3)	34(5)	31(4)	36(7)	18(8)	17(9)
Moderate symptoms	67(1)	39(2)	37(3)	—	48(2)	44(4)	42(2)	72(5)	33(4)	27(4)
Severe symptoms	—	55(2)	59(3)	—	55(1)	49(2)	—	74(1)	38(7)	29(2)
Survival time in days and (in parentheses) number of animals	—	55(1)	48(3)	—	56(2)	52(3)	32(5)	80(7)	40(8)	35(8)

^a The ration used in this experiment was supplemented with the following vitamins, added per kilogram of diet: thiamin, 2 mg.; riboflavin, 4 mg.; and calcium pantothenate, 20 mg.

^b The ration used was supplemented with the following vitamins, added per kilogram of diet: thiamin, 10 mg.; riboflavin, 10 mg.; and calcium pantothenate, 40 mg. The animals in group (a) received in addition choline (1.0 g. of choline chloride per kilogram of diet).

¹ All rats in this series were changed to the 45% casein diet on the 77th day. Acro-dynia developed in about one week, and the animals died in three weeks.

² One rat in this group was given 8 μ g. of pyridoxin daily; it recovered.

³ Administration of 8 μ g. of pyridoxin to one of the rats was curative. One rat in this series died prematurely of starvation.

⁴ The surviving rats in this experiment were given 3 μ g. of pyridoxin daily, with gradual cure of the symptoms and slight growth.

⁵ One rat in this group was alive and without symptoms on the 166th day.

⁶ One rat in this series was excluded; it lived 98 days.

that Sullivan (7) observed an excretion of indolethylamine in the urine in pellagra, which may indicate a disturbance of tryptophan catabolism. Finally, Voris and Moore (8) have shown in paired feeding experiments that rats receiving pyridoxin gained more weight than their pyridoxin deficient pair-mates, and that the gains were associated with an increase of water and protein in the tissues, and with no effect on body fat.

It should be mentioned at this point that a strain difference in rats as regards their susceptibility to pyridoxin deficiency has been observed in this laboratory. This will be the subject of a future communication.

SUMMARY

A study has been made of the acrodynia developing in rats maintained on pyridoxin deficient rations containing varying amounts of protein. It has been found that the time of appearance and the severity of the symptoms are influenced by the protein intake. In rats that received diets high in casein, the acrodynia appeared at an earlier period and was more severe than in those on diets low in casein. These findings suggest a relationship between pyridoxin and protein metabolism.

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Photochemical Reaction of Iodine with Carotenoids¹

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The *cis-trans* isomerization of carotenoids has recently been reviewed in considerable detail (1). In their work, Zechmeister and co-workers have employed catalytic amounts of iodine in the presence of light to promote isomerization. They have described the resultant products as an equilibrium mixture of *cis-trans* isomers. This led the authors to investigate catalytic iodine-light isomerization as a possible analytical tool for spectroscopic analysis of naturally occurring mixtures of carotenoid stereoisomers (or those formed during extraction procedure) as a simple mixture of a small number of isomeric sets (α - and β -sets, for example), treating each set analytically as a single component (2).

Difficulty was soon experienced in obtaining reproducible results on such mixtures, even when pure α - or β -carotene was used as starting material; therefore, a more detailed investigation was undertaken of the factors influencing the amount of spectroscopic change brought about by iodine-light isomerization. A study of the members of the α -carotene stereoisomeric set has already been reported by two of the authors (3).

METHODS

Hexane was the solvent employed unless otherwise noted. Solvents were redistilled over NaOH before use, except hexane (Viking Distributing Co.—Normal hexane, b.p. 65–67° C.) which was treated exhaustively (agitation for three weeks at room temperature) with alkaline permanganate before distillation. After this treatment the permanganate was not appreciably reduced in 1 to 2 days' agitation with hexane. Temperatures were maintained at 25–27° in a light-tight, air thermostat during isomerization. Absorption measurements were determined with a photo-

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electric spectrophotometer, the performance of which has been previously described (4). Errors were 1% or less. (Note the excellent agreement between the spectroscopic results on all-*trans* α - and β -carotenes in hexane solution measured with a Hilger optical system (4) and those more recently reported by Zechmeister and Polgár (5), as measured with a Beckman spectrophotometer.)

In this work 4720 and 4780 Å were employed to follow the course of isomerization of α - and β -carotenes, respectively. These wave lengths are near the corresponding longest wave length maxima of both the all-*trans* parent isomers and the isomerization mixtures. Absorption was also measured at 4000 Å for α -carotene, since absorption at this wave length does not change appreciably during initial stages of iodine action (3).

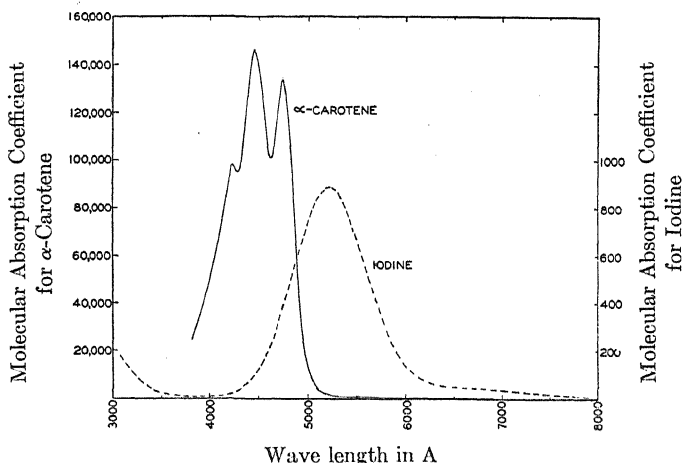


FIG. 1

Absorption Spectra of α -Carotene and Iodine in Hexane Solution

$\log I_0/I = \alpha c l$; α = molecular absorption coefficient; c = concentration in mols per liter; l = cell length in cm.

α -Carotene was employed in most of these studies because stock solutions in hexane remained spectroscopically constant at refrigerator temperatures ($+4^\circ$) longer than solutions of β -carotene, which showed appreciable changes in 24 hours.

It appeared desirable to employ monochromatized radiation in the study of effect of wave length on this reaction. The most suitable source was the 5460 Å line from a mercury arc (Type H-4) filtered with Corning filters 351 (2 mm.), 430 (4 mm.) and 512 (5 mm.). This wave length is excellent because it occurs near the absorption maximum of iodine and in a region of relatively low carotenoid absorption, especially for α -carotene (see Fig. 1). At 5460 Å the molecular absorption coefficients of α -carotene and iodine are practically equal, being 699 and 720, respectively. At 4358 Å, isolated from the mercury arc spectrum with Corning filters 038 (2.3 mm.)

and 511 (2.5 mm.), the corresponding absorption values are 117,000 and 60 with a ratio of 1950. In previous work (6) it had been found that the mercury arc employed with these filters produced nearly equal irradiancies at these two wave lengths. The use of these lines permits an approximate 2000-fold change in the relative amounts of radiation absorbed by iodine and carotene.

Since illumination greater than 36 foot candles (measured with a Weston Illumination Meter, Model 603) could not be conveniently obtained with the filtered mercury arc, higher illumination, up to 500 f.c., was obtained with a 60-watt, unfiltered, incandescent filament lamp.

Isomerization studies were conducted in a Pyrex or Corex D absorption cell (usually 1 cm. in length) in which carotene solution and iodine dissolved in the same solvent were mixed. These operations were carried out with a minimum exposure to light.

Iodine concentrations are expressed in terms of c , which was arbitrarily made to coincide with a concentration such that the ratio of carotenoid-to-iodine concentrations would be similar to those used in many experiments by Zechmeister, *et al.* (5, 7, 8). $1\ c = 0.05$ mg. of I_2 per liter of carotenoid solution. Carotenoid concentrations (usually 4 to $5.6 \times 10^{-6} M$) were considerably lower than those employed by Zechmeister because of the difference in method of measurement of the progress of isomerization. The molar ratio of initial carotenoid-to-iodine concentrations is reported as r . The molecular absorption ratio represents the proportion of light absorbed by carotene with respect to iodine and was calculated by multiplying the molar ratio by the ratio of molecular absorption coefficients at the wave length concerned.

Photochemical Nature of the Iodine Action

The following experiment confirms the necessity of light in causing the action of iodine on α -carotene (1, 7, 9).

Conditions	Decrease in absorption after 24 hours	
	4000 Å per cent	4720 Å per cent
$1\ c\ (r = 27)$, darkness	<1	2.8
$0\ c$, 36 f.c.	<1	4.7
$1\ c\ (r = 27)$, 36 f.c.	14.5	40.0

No greater changes occurred in the presence of iodine in the dark than could be accounted for by thermal considerations alone.

Factors Influencing the Iodine Action

The effects of wave length of radiation, illumination, solvent, iodine concentration, and carotene concentration on the absorption changes accompanying the photochemical action of iodine on carotene were studied.

1. *Wave length of radiation.* After establishment of the photochemical nature of the reaction, an attempt was made to determine the wave length threshold. This was done employing filtered incandescent radiation and a temperature of 20°. β -Carotene was used for these studies (47 c, $r = 0.5$) with Corning filters 242 (2 mm.) and 254 (3.5 mm.), radiation of wave lengths shorter than 8000 Å was effectively eliminated. The cell filled with solution was placed 35 cm. from a 60-watt bulb in a light-tight box with the filter set as a window (the illumination could only be measured without the filter and under such conditions it was 36 f.c.). No decrease in absorption greater than 1% at 4780 Å was found after 5 hours. Under similar conditions, using filters 242 and 502 (4 mm.) which restricted the radiation to wave lengths above 7000 Å, a decrease of 7% occurred in 3 hours. These experiments indicated the photochemical threshold to be above 7000 Å.

Comparisons of rates were made with filtered radiation from the mercury arc, using lines 5460 and 4358 Å separately on α -carotene solutions. With 5460 Å at 12 f.c. (1 c, $r = 27$; molecular absorption ratio = 27) a decrease of 17% in absorption at 4720 Å occurred in 80 minutes. When the 4358 Å line was used (molecular absorption ratio = 5300) no change occurred in the same time; however, when an absorption ratio of 104 (51 c) was used, absorption decreased. These experiments indicate that the radiation absorbed by the iodine is more effective than that absorbed by carotene in bringing about the spectroscopic changes observed.

2. *Illumination.* α -Carotene was studied over a 6-fold range of illumination values (6 to 36 f.c. filtered 5460 Å mercury line; 1 c, $r = 27$). The initial rate of absorption decrease at 4720 Å increased with illumination in this range. An increase in rate with increasing illumination was also observed with β -carotene ($\frac{1}{2}$ to 12 f.c. filtered 5460 Å mercury line; 47 c, $r = 0.5$).

3. *Solvent.* Most of our early experiments were done using hexane as a solvent. In attempting to study quantitatively various relationships of illumination values and iodine concentrations, numerous discrepancies led us to suspect that the catalytic action of iodine did not continue indefinitely. An experiment was designed to permit the measurement of absorption of both iodine and α -carotene during a prolonged exposure to light (135 c, $r = 0.2$). Absorption measurements were made at 5200 Å (maximum for I_2 ; molecular absorption ratio = 0.32) and at 4720 Å (near maximum for α -carotene; molecular absorp-

tion ratio = 66). Results are presented in Fig. 2. A high iodine concentration (and consequently a low r value) was chosen to permit measurements of the iodine concentration by use of wave length 5200 Å. Measurements at 4720 Å indicated a rapid change in carotene. During the same time measurements at 5200 Å showed that molecular iodine was disappearing from solution. Absorption by both substances finally approached zero at similar rates. In a control solution of iodine in hexane with no carotenoid, iodine also disappeared but at a slower rate than in the presence of carotene.

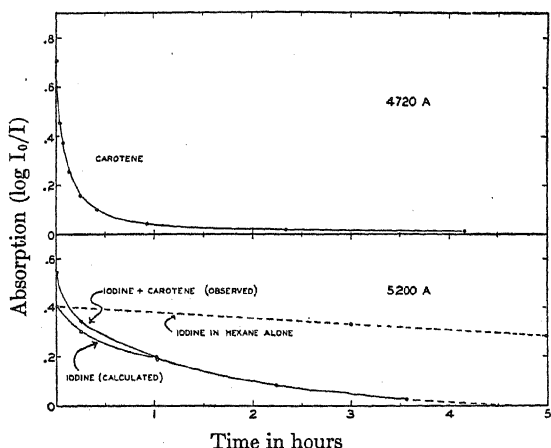


FIG. 2

Absorption Changes of α -Carotene and Iodine during Iodine-Light Treatment in Hexane

500 f.c. incandescent illumination; 135 c, $r = 0.2$

Two other solvents, iso-octane (2,2,4-trimethyl pentane) and cyclohexane, were then compared with hexane in a study of the rate of disappearance of iodine from them during irradiation. A solution of 135 c was illuminated with 500 f.c. incandescent illumination for 24 hours. The rate of disappearance of iodine was followed by absorption measurements at 5200 Å. At the end of the experiment 8.6, 92.0, and 96.0% of the original iodine remained in hexane, iso-octane, and cyclohexane, respectively. In the dark, iodine did not disappear from such solutions.

Additional confirmation of the removal of iodine from catalytic influence was obtained by the addition of all-*trans* α -carotene to solutions which had been freshly isomerized by iodine-light (1 c, $r = 14$). The results for the three solvents are presented in Fig. 3. When additional carotene was added after about 40 minutes illumination, the absorption did not change in hexane but did decrease in iso-octane and cyclohexane. The failure to obtain additional change when fresh all-*trans* carotene was added to the hexane solution indicates that all the iodine had been removed by reaction with the solvent and/or carotene. The additional experiment (dotted line) demonstrates con-

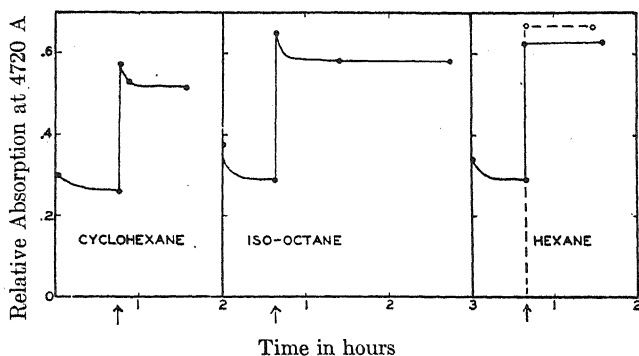


FIG. 3

Effect of Solvent on Removal of Iodine from Catalytic Influence as Shown
by Isomerization of α -Carotene

500 f.c. incandescent illumination; 1 c, $r = 14$. All-*trans* isomer was added at time indicated by arrow; dotted line indicates experiment in the initial absence of carotene

clusively the loss of iodine during the 40 minutes illumination before addition of carotene. Thus the removal of iodine by solvent may cause a steady state which is really an artifact.

After filtration through 20 inches of silica gel (Davco 695150, manufactured by the Davidson Chemical Corporation, Baltimore, Maryland), a procedure suggested by M. M. Graff (10), hexane did not absorb iodine as rapidly as before, as shown by the fact that carotene absorption changes occurred after 45 minutes illumination of an iodine-hexane mixture and subsequent addition of carotene. The absorption change was $\frac{1}{4}$ that observed with iso-octane (Fig. 3).

4. *Iodine concentration.* Results obtained in iso-octane, holding the α -carotene ($5.6 \times 10^{-6} M$) concentration constant and varying the concentration of iodine over a 10-fold range ($\frac{1}{2} c$ to $5 c$) are represented in Fig. 4. After an initial rapid change, probably mainly *cis-trans* isomerization, the absorption at 4720 A approached zero, the rate being dependent on the iodine concentration. It should be noted that carotene absorption at 4720 A approached zero even though there

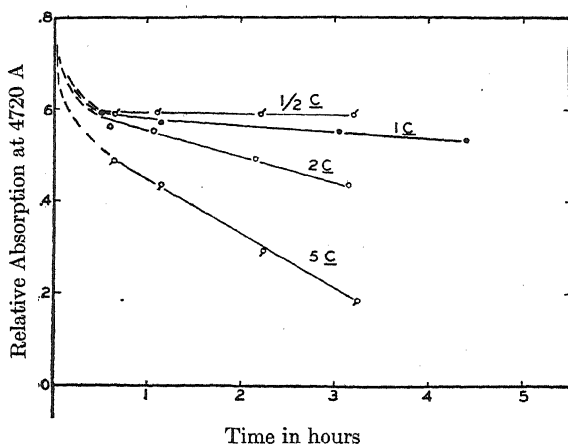


FIG. 4

Effect of Iodine Concentration on Absorption Change in Iso-octane

500 f.c. incandescent illumination; α -carotene concentration = $5.6 \times 10^{-6} M$

c	r
$\frac{1}{2}$	54
1	27
2	13.5
5	5.4

was present less than one atom of iodine per molecule of carotene. In the $2 c$ and $5 c$ experiments the presence of free iodine was demonstrated after 15 hours. In studying the effects of iodine concentration, removal of iodine by reaction with the solvent or the carotene or by adsorption on glass surfaces should be considered.

Removal of iodine by thiosulfate and addition of a smaller concentration of iodine did not result in reversal of the reaction, but instead in continued decrease of absorption.

Studies of the effect of iodine concentration show that iodine action is not limited to a catalytic role resulting only in an equilibrium mixture of *cis-trans* isomers and serve to emphasize the importance of secondary reactions recently reported by Zechmeister (1). Neither can iodine act only as one component of an equilibrium involving molecular iodine itself.

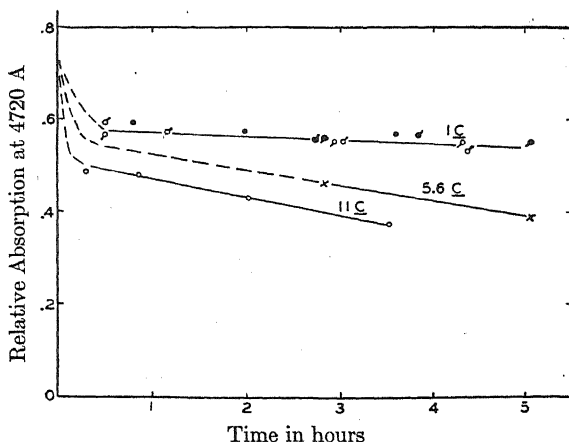


FIG. 5

Effect of Carotene Concentration and Ratio of Carotene-to-Iodine Concentrations on the Absorption Change in Iso-octane

500 f.c. incandescent illumination

α -Carotene Concentration		r
●	$1.4 \times 10^{-6} M$	6.7
○	$2.8 \times " "$	13.5
◐	$5.6 \times " "$	27
◑	$3.2 \times 10^{-5} M$	151
●	$6.2 \times " "$	297
<hr/>		
×	$3.2 \times 10^{-5} M$	27
○	$6.2 \times " "$	27

5. *Carotene concentration.* Variation of α -carotene concentration over a 44-fold range (1.4×10^{-6} to $6.2 \times 10^{-5} M$) while holding iodine concentration constant (1 c) led to the results shown in Fig. 5. In this figure all absorption values have been calculated to the same relative absorption at zero time. Thus the figure compares the frac-

tion of the carotene changed at different carotene concentrations. This fraction is almost constant in the range studied.

Fig. 5 also includes two experiments in which *both* the iodine and carotene concentrations were relatively high (α -carotene = $6.2 \times 10^{-5} M$, 11 c, $r = 27$; α -carotene = $3.2 \times 10^{-5} M$, 5.6 c, $r = 27$). The effect of carotene concentration is shown by comparison of curves for 5 c and 5.6 c in Figs. 4 and 5, in which the rates of absorption change are considerably different. In Fig. 5 the fraction of the carotene changed increases with increasing iodine concentrations, even though the ratio of carotene-to-iodine concentrations is held constant ($r = 27$). In this experiment as in Fig. 4, the effect of absolute iodine concentration is clear and emphasizes that control of only the ratio of iodine-to-carotene concentrations will not insure reproducible results over a wide range of carotene concentrations.

General Considerations

The magnitude of secondary changes resulting in gross decreases in absorption and the dependence of such changes on the absolute iodine concentration and other factors seems effectively to preclude the use of iodine-light isomerization methods for the spectroscopic analysis of mixtures of carotenoids from natural sources. A further complication in practice is the inevitable inclusion of materials other than carotenoids in plant extracts which might absorb large amounts of iodine, thereby quickly removing iodine from its catalytic role.

SUMMARY

The photochemical action of iodine on α - and β -carotenes in solution was studied in relation to several factors. The photochemical threshold occurs in the spectral region above 7000 Å. At low values, illumination may limit the rate of absorption decrease. The effective radiation is that absorbed by iodine rather than by carotene.

The photochemical action of iodine on the hydrocarbon solvent (or impurity) may be the cause of spurious results because it effectively removes iodine from catalytic influence on carotene. Cyclohexane and iso-octane are better solvents than hexane for studies of isomerization because iodine disappears from them much more slowly.

Spectroscopic observations were used to follow the course of the reaction. An initial rapid decrease of absorption at 4720 Å for α -caro-

tene, largely due to isomerization, is followed by a much slower decrease, which may, under some conditions, involve gross destruction of the carotene.

The absolute concentration of iodine is more significant in determining the fractional change of absorption than is the carotenoid-to-iodine ratio of concentrations.

It is concluded that although an equilibrium mixture of carotenoid stereoisomers may be formed catalytically through the photochemical action of iodine, further complications arise from an irreversible photochemical reaction of iodine with carotene; the over-all mixture is thus very complex. The use of such mixtures as an aid in quantitative spectroscopic analysis of plant extracts is not very promising.

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Factors Affecting the Determination of Biotin by Means of *Lactobacillus Casei* *

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INTRODUCTION

In 1942, Shull, Hutchings, and Peterson proposed the use of *Lactobacillus casei* as an assay organism for the microbiological determination of biotin. Some investigators who have used this method have encountered two types of difficulties; one, a high blank (acidity) in the absence of added biotin and, two, a low acidity in the presence of biotin. These difficulties can be traced back to the hydrolyzed casein used to supply the required amino acids and to the yeast filtrate supplement added to furnish the norite eluate factor (which is closely related to, if not identical, with folic acid and vitamin B₁₂, 1). In treating the hydrolyzed casein and yeast filtrate the problem is to remove the accompanying biotin and leave an adequate supply of the eluate factor. A procedure to accomplish these two objectives has been described by Shull and Peterson (2, 3).

The difficulty could of course be met by using pure amino acids and pure eluate factor. Except for the cost, the use of pure amino acids seems satisfactory, but use of the pure growth factor may not be desirable because of the omission of small amounts of stimulatory substances ordinarily supplied by the yeast supplement. Similar stimulatory substances may be contained in the sample under assay and cause a response that is erroneously attributed to biotin.

The effects of a number of factors that have been reported to influence the activities of *L. casei* have also been tested. These include the

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use of methyl ester of biotin, certain metallic salts, lactose, thymine, and vitamin B₆, in the biotin assay.

EXPERIMENTAL

Basal Media

The composition and preparation of the medium as described by Shull *et al.* (2, 3) was followed unless otherwise indicated. Table I lists the ingredients of the

TABLE I
Constituents of the Basal Media

	No. 1 <i>per cent</i>	No. 2 <i>per cent</i>	No. 3 <i>per cent</i>	No. 4 <i>per cent</i>
Peroxide treated hydrolyzed casein	0.50	0.50	0.50	†
Peroxide treated norit yeast filtrate	0.15	0.15	0.30	†
Tryptophan	0.0075	0.0075	0.0075	0.0075
Cystine	0.01	0.01	0.01	0.01
Asparagine	0.02	0.02	0.02	0.02
Glucose	1.00	1.00	4.00	1.00
Sodium acetate	1.00	0.60	1.50	1.00
Mineral salts	*	*	*	*
	<i>mg.</i> <i>per cent</i>	<i>mg.</i> <i>per cent</i>	<i>mg.</i> <i>per cent</i>	<i>mg.</i> <i>per cent</i>
Pyridoxin	0.02	0.02	0.04	0.02
Pantothenic acid	0.02	0.02	0.04	0.02
Riboflavin	0.01	0.01	0.02	0.01
Nicotinic acid	0.10	0.10	0.20	0.10
p-Aminobenzoic acid	0.01	0.01	0.02	0.01
Thiamin	—	0.01	—	0.01
Adenine	1.00	1.00	1.00	1.00
Guanine	1.00	1.00	1.00	1.00
Xanthine	—	2.00	—	2.00
Uracil	—	2.00	—	2.00

* Per cent in final medium: K₂HPO₄ 0.05, KH₂PO₄ 0.05, MgSO₄·7H₂O 0.02, NaCl 0.001, FeSO₄·7H₂O 0.0008, MnSO₄·4H₂O 0.008.

† Source of nitrogen and eluate factor supplied as indicated in text and tables.

several media. Medium 1 is that described by Shull *et al.* (2, 3). Medium 2 approximates the medium described by Landy and Dicken (4). The stock solutions employed in making Medium 1 were used plus xanthine, uracil, and thiamin. The amount of sodium acetate was reduced from 1% to 0.6%. Medium 3, an enriched medium patterned after Light and Clarke (5), contained 50% more acetate, twice as much vitamins and four times as much glucose as Medium 1. Other constituents were

used in the same amounts as in Medium 1. Medium 4 was designed for use with pure vitamin Bc. It omits the yeast filtrate and leaves out the hydrolyzed casein, and this was replaced by amino acids.

Standards

Crystalline SMA biotin and hydrolyzed biotin methyl ester from Merck were used as standards. A standard curve with a blank of 2.2 cc. or less of 0.1 *N* acid per 10 cc. of medium, rising smoothly to 9 cc. or more with maximum biotin addition was set as a requirement. As a further check on the procedure a reference material which had been very carefully analyzed for its biotin content was included in each assay.

Utilization of Biotin Methyl Ester

In the original paper on the biotin assay, Shull, Hutchings, and Peterson (2) state that *L. casei* is unable to use the methyl ester. Stokes and Gunness (6) reported that, although the cell growth was slower with the ester than with the free acid, after 3 days incubation there was no appreciable difference in titratable acid between cultures containing either the free acid or the methyl ester. Because of these conflicting reports, we have repeated and extended the tests with the two compounds and found that under certain conditions there was little or no utilization of the methyl ester while under other conditions there was 100% utilization.

A methanol solution of the unhydrolyzed biotin methyl ester, 0.4 γ /cc., was kept under refrigeration, and a fresh dilute water solution was used for each experiment unless otherwise indicated. Methyl ester stored in a water solution for one month gave an acid production of 10 cc. per tube as compared to 8 cc. when fresh methyl ester was used. Hydrolysis on standing, although slow, was definite. Early results with Medium 1 showed little or no acid production in tubes containing up to 800 $\mu\gamma$ per tube of methyl ester, calculated as free acid. With Medium 2, at pH 6.8, up to 35% utilization was found. This result appeared to be related to the pH of the medium rather than to differences in the basal medium. To determine whether increased utilization was due to the hydrolysis of the methyl ester by autoclaving at the high pH, the methyl ester solution was sterilized by means of a Seitz filter and added aseptically to the sterile basal. Table II shows no significant difference in the amount of acid produced with different methods of sterilization or different basal media. Results with filter sterilization varied both above and below results with heat steriliza-

TABLE II

Effect of pH, Method of Sterilization, and Medium on Utilization of Biotin Methyl Ester

Methyl ester calculated as $\mu\gamma$ biotin/10 cc.	pH 5.6			pH 6.8		
	HH*	UH	UF	HH	UH	UF
Medium 1						
0	2.4	2.6	2.5	1.1	1.0	1.1
100	4.1	2.3	2.6	3.7	1.5	1.2
200	6.9	2.6	2.9	5.5	1.6	1.5
400	8.6	2.7	3.6	7.1	2.6	2.5
800	9.8	3.3	4.8	9.0	5.8	4.7
Medium 2						
0	2.7	2.7	2.0	0.8	0.8	1.0
100	5.3	2.7	2.1	5.2	1.4	1.2
200	8.2	2.8	2.1	6.2	3.4	2.2
400	9.8	2.7	2.8	8.5	5.6	3.8
800	10.5	3.4	4.6	8.4	7.8	7.1

* HH—Hydrolyzed, heat sterilized.

UH—Unhydrolyzed, heat sterilized.

UF—Unhydrolyzed, filter sterilized.

tion. At the highest level of biotin there was much greater utilization of the ester at pH 6.8 than at pH 5.6. Uninoculated filter-sterilized blanks were constantly low, indicating the absence of contamination.

The effect of pH on the utilization of the biotin methyl ester was studied further. Table III summarizes a set of these results. Utilization of the ester as judged by acid production was greatly favored by the higher pH and, at the highest biotin level (800 $\mu\gamma$), was practically equal to that reached with the free acid. In most cases acid production was not compete with a three-day incubation period. The increased acid production with five-days incubation was not due to spontaneous hydrolysis of the biotin ester on standing in the medium because similar tubes, inoculated after standing 3 days at incubation temperature, showed no greater acid production than the tubes inoculated at once.

The use of an enriched medium, No. 3, patterned after Light and Clarke (5) resulted in no appreciable increase in the utilization of biotin methyl ester (Table IV). With filter sterilization from 25 to

TABLE III

Effect of pH and Incubation Time on Utilization of Biotin Methyl Ester
cc. 0.1 N acid per 10 cc. of culture, Medium No. 1

Biotin $\mu\gamma$ /10 cc.	Standard titer	pH 5.6		Standard titer	pH 6.8	
		Methyl ester Titer	per cent*		Methyl ester Titer	per cent*
3-day incubation						
0	2.0			1.3		
200	6.0	2.1	2.0	5.8	5.0	70
400	8.0	2.8	7.5	7.8	7.0	77
800	9.3	5.3	18.8	9.1	9.1	100
5-day incubation						
0	2.7			2.2		
200	8.4	4.9	22	7.9	7.6	80
400	10.0	7.7	34	9.5	9.4	99
800	10.7	9.5	42	9.6	9.3	97

* Per cent biotin activity as calculated from standard curve.

TABLE IV

*Effect of Enriched Medium on Acid Production with Biotin and its Methyl Ester**
cc. 0.1 N acid per 10 cc. culture, Medium 3

Biotin $\mu\gamma$ /10 cc.	Hydrolyzed ester, titer	Methyl ester		Filter sterilized Titer	per cent†
		Heat sterilized Titer	per cent†		
0	3.5	3.3	0	1.0	
				(uninoculated)	
100	8.5	5.3	40	Not run	
400	12.5	11.2	75	8.3	25
800	15.0	13.7	71	10.3	28
1600	19.6	17.1	74	15.0	50
3200	22.7	21.7	75	19.8	50

* Initial pH of medium, 6.8; incubation, 3 days.

† Biotin activity as calculated from standard curve.

50% biotin activity was found while with heat sterilization, possibly because of hydrolysis during sterilization, the availability reached 75%.

An experiment in which the microorganism was transferred after one-day and two-day growth in tubes containing Medium 1 and 800 $\mu\gamma$ of methyl ester resulted in no increase in acid production and, hence, indicated no adaptation of the microorganism to the methyl ester. Stokes and Gunness (6) report no change with 9 serial transfers.

Different types of inoculum and their effect on utilization of methyl ester were studied as follows:

Inoculum I. Difco yeast broth (0.5% Difco yeast extract, 0.6% NaOAc, 0.5% glucose, 0.05% mineral salts) was inoculated from a stab and incubated 20–24 hours at 37°. The cells were centrifuged and the supernatant liquid was poured off. The cells were resuspended in 10 cc. of sterile water and one drop per 10 cc. of Medium 1 was used. The initial pH was 5.6, and the incubation period was 3 days.

Inoculum II (Standard method). One drop of Inoculum I was suspended in 10 cc. of sterile water. One drop of this was used per tube. Titer was the same regardless of number or size of drops as reported by Shull and Peterson (3).

Inoculum III. As described by Shull *et al.* (2), this inoculum was started two days before the assay was to be set up. A transfer was made from a stab culture to a tube of Difco yeast extract broth and incubated at 37° for 24 hours. A 2% inoculum of this was then transferred to sterile biotin-free basal medium, and the culture was incubated for 15 to 20 hours at 37°. One drop of the suspension was used per tube.

A high blank was noted with Inoculum I, and the titer increased with size of drop per tube (Table V). Inoculum I gave irregular in-

TABLE V

Effect of Type of Inoculum on Titer with Free Acid (F.A.) and Methyl Ester (M.E.) of Biotin

cc. of 0.1 N acid per 10 cc. of culture, Medium 1

μ y Biotin/ 10 cc.	Inoculum I		Inoculum II		Inoculum III	
	F.A.	M.E.	F.A.	M.E.	F.A.	M.E.
0	3.4	3.7	2.0	2.2	2.2	2.2
200	7.1	5.5	6.4	2.3	7.4	2.4
400	7.8	7.1	7.4	2.7	9.1	3.1
800	9.7	8.1	10.2	4.3	10.1	5.9

creases in acidity at the several levels of biotin. The response was almost as high with the ester as with the free acid but in neither case was maximum acidity reached at the highest level of biotin. Inocula II and III gave low blanks and were independent of drop size. Inoculum III gave a regular and maximum acidity with the biotin free acid, but an irregular and low response to the biotin methyl ester. Since inocula I and II contained identical cells, it was the number of cells that determined the results.

From the data in the preceding tables, it is evident that acid production with the methyl ester of biotin is highly irregular. Some of the irregularity was found to be due to basal medium, biotin level, initial pH, inoculum, and incubation period. It is clearly advisable to have biotin in the free acid form when assaying materials for this vitamin or where using it as a vitamin supplement.

Sources of Nitrogen

Variations in the steepness of standard curves on Medium 1 were noted and were found to be related in part, at least, to different casein preparations. Casein hydrolyzates made according to Shull *et al.* (2) had low blanks, high maximum titers, but varied considerably in steepness. Casein hydrolyzates made in a somewhat different manner were also tested, and the results are tabulated in Table VI. The F

TABLE VI

Comparison of Acid Production with Different Sources of Nitrogen
cc. 0.1 N acid per 10 cc. of culture, Medium 1

μ g Biotin/ 10 cc.	Casein hydrolyzates						Amino Acids*
	F	S	K	A-1	A-2	SMA	
0	2.0	2.1	2.1	2.3	6.8	2.1	2.3
100	4.9	5.9	3.6	4.8	7.3	4.0	3.0
200	6.4	7.9	5.4	6.0	8.0	5.6	4.5
400	7.4	9.8	7.5	7.7	8.8	7.5	6.8
800	10.2	10.8	9.2	10.1	10.4	9.4	10.4
1600	Not run		11.0	11.8	10.9	11.6	9.2

* 2 mg. per 10 cc. of following: *dl*-alanine, *dl*-leucine, *dl*-isoleucine, *dl*-serine, *dl*-threonine, *l*-tyrosine, *dl*-phenylalanine, *dl*-lysine, *dl*-methionine, *dl*-glutamic acid, *dl*-valine, *dl*-aspartic acid, *dl*-cystine, *d*-arginine, *l*-histidine, *l*-tryptophan.

and S caseins were acid hydrolyzed under pressure and peroxide treated, according to Shull *et al.* (2). The K preparation was hydrolyzed in the same way, but was treated with norit. This preparation seemed slightly inferior to the S and F preparations due to the removal by norit of some stimulatory factors not destroyed by the peroxide. The A-1-hydrolyzate was treated with Darco G-60 twice and was practically colorless. The A-2 was hydrolyzed by refluxing the casein with HCl for 10 hours, then treating with Darco G-60, and still retained considerable color. This treatment evidently did not destroy the biotin completely as indicated by the high blank. The SMA-hydrolyzate was a commercial product, and the curve was comparable to that obtained with the K preparation.

Included in Table VI is a series in which the casein hydrolyzate was replaced by the mixture of amino acid required by *L. casei* as reported by Hutchings and Peterson (7). Acid production at low and intermediate levels of biotin was lower than with hydrolyzed casein, which suggests that casein hydrolyzate provides a stimulatory factor

as well as nitrogen supply. At high levels of biotin satisfactory acidities were obtained with the amino acid mixture.

Addition of Other Compounds

The effect on the biotin standard of adding metallic salts, lactose as a source of carbohydrate, and thymine to basal Medium 1 was studied. Chattaway *et al.* (8) observed differences in growth curves with different casein hydrolyzates and attributed it to variation in salt content. Luckey *et al.* (9) noted increased growth with *S. lactis* with increased amounts of KH_2PO_4 . In our experience additional Ca^{++} (0.001% $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$) and Zn^{++} ions (0.008% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) had no noticeable effect while 0.002% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ completely inhibited acid production. With 0.36% K^+ (KCl) there was no effect and with 0.72% there was a slight inhibition.

Sullivan *et al.* (10) recommend the use of lactose as the carbohydrate when analyzing cheese for vitamins with *L. casei*. Lactose was found to be a satisfactory carbohydrate for *L. casei* but some stimulation, possibly due to biotin or stimulating substances adsorbed on the lactose, was noted at low levels of biotin.

Thymine, which can substitute in part for Vitamin B_6 , had no effect on the biotin standard curve.

Replacement of Yeast Supplement by Vitamin B_6

The purpose of the yeast supplement in the biotin assay is to furnish the norit eluate factor required by *L. casei*. Since crystalline Vitamin B_6 can be obtained from yeast extracts and is known to affect the growth, *L. casei* experiments were set up to see if Vitamin B_6 could replace the eluate factor in the biotin assay method.* In Table VII are given the acidities obtained with yeast filtrate, Vitamin B_6 , and a combination of the two. With B_6 alone, the acidities are somewhat lower than those with yeast filtrate after 3 days' incubation, but are not significantly different after 5 days' incubation. The yeast filtrate evidently supplies something more than crystalline B_6 , which suggests that a stimulatory substance contained in yeast filtrate is slowly synthesized by *L. casei*. Since acid production with both yeast filtrate and vitamin B_6 is not appreciably different from that with yeast

* We are indebted to Dr. A. D. Emmett of Parke, Davis, and Co. for the crystalline vitamin B_6 used in these experiments and extend to him our sincere thanks.

TABLE VII

*Effect of Replacing Yeast Filtrate (Y.F.) with Vitamin B₆ on Acid Production**
cc. of 0.1 N acid per 10 cc. of culture

$\mu\gamma$ Biotin/ 10 cc.	3 day			5 day		
	1 cc. Y.F.	2 m γ B ₆	1 cc. Y.F. + 1 m γ B ₆	1 cc. Y.F.	2 m γ B ₆	1 cc. Y.F. + 1 m γ B ₆
0	1.3	0.9	1.0	1.3	1.0	1.6
100	5.1	4.5	4.9	†	†	†
200	5.8	5.6	6.1	7.0	6.4	6.9
400	7.7	7.1	8.3	8.4	9.0	8.5
800	9.0	8.3	8.8	9.4	9.4	9.5

* Medium 4, pH 6.8.

† Not run.

filtrate alone, it indicates that the latter contains all the vitamin B₆ or its equivalent that is required by the organism.

Synthetic Medium

Since amino acids have been successfully substituted for hydrolyzed casein (7) and vitamin B₆ can be used in place of yeast filtrate, a completely synthetic basal medium was tested. Medium 4 in Table I was used with the following changes and additions. All vitamins were doubled. Two m γ of vitamin B₆ were added per 10 cc.; sodium acetate, 0.6% instead of 1%; amino acids, 2 mg. per tube. The assay was run at pH 6.8 and four day incubation resulted in satisfactory acid production as shown in Table VIII. A heavier inoculum had the

TABLE VIII

Effect of Time and Inoculum Strength on Acid Production with Medium of Known Constituents

cc. of 0.1 N acid per 10 cc. of culture, Medium 4

$\mu\gamma$ Biotin/ 10 cc.	Run No. 1	72 hours			90 hours		110 hours
		2	2*		1	2	2
0	0.9	0.9	1.1		1.1	0.8	0.9
100	3.9	3.8	4.2		4.9	4.5	5.6
200	5.5	5.8	6.3		6.7	6.6	7.7
400	6.7	7.4	8.2		8.6	8.4	9.2
800	7.6	8.4	9.2		9.2	9.6	9.8

* Heavier inoculum used. See text.

same effect on acid production as a longer incubation period. This inoculum was prepared by suspending 1 cc. instead of 1 drop of cells in 10 cc. of sterile water, and 1 drop was used per tube. This inoculum is twenty times the inoculum used in the other tubes.

The results obtained with the synthetic medium for biotin suggest that the same medium could be used for the assay of riboflavin, pantothenic acid, and nicotinic acid. However, because of the longer time required and the possibility of other substances present in natural materials affecting the results, further experiments would be needed before adopting a synthetic medium for such assays.

SUMMARY

To clear up some difficulties that arise in assays of biotin, the utilization of the methyl ester of biotin by *Lactobacillus casei* has been studied under different conditions of pH, composition of medium, sterilization, incubation, and inoculum.

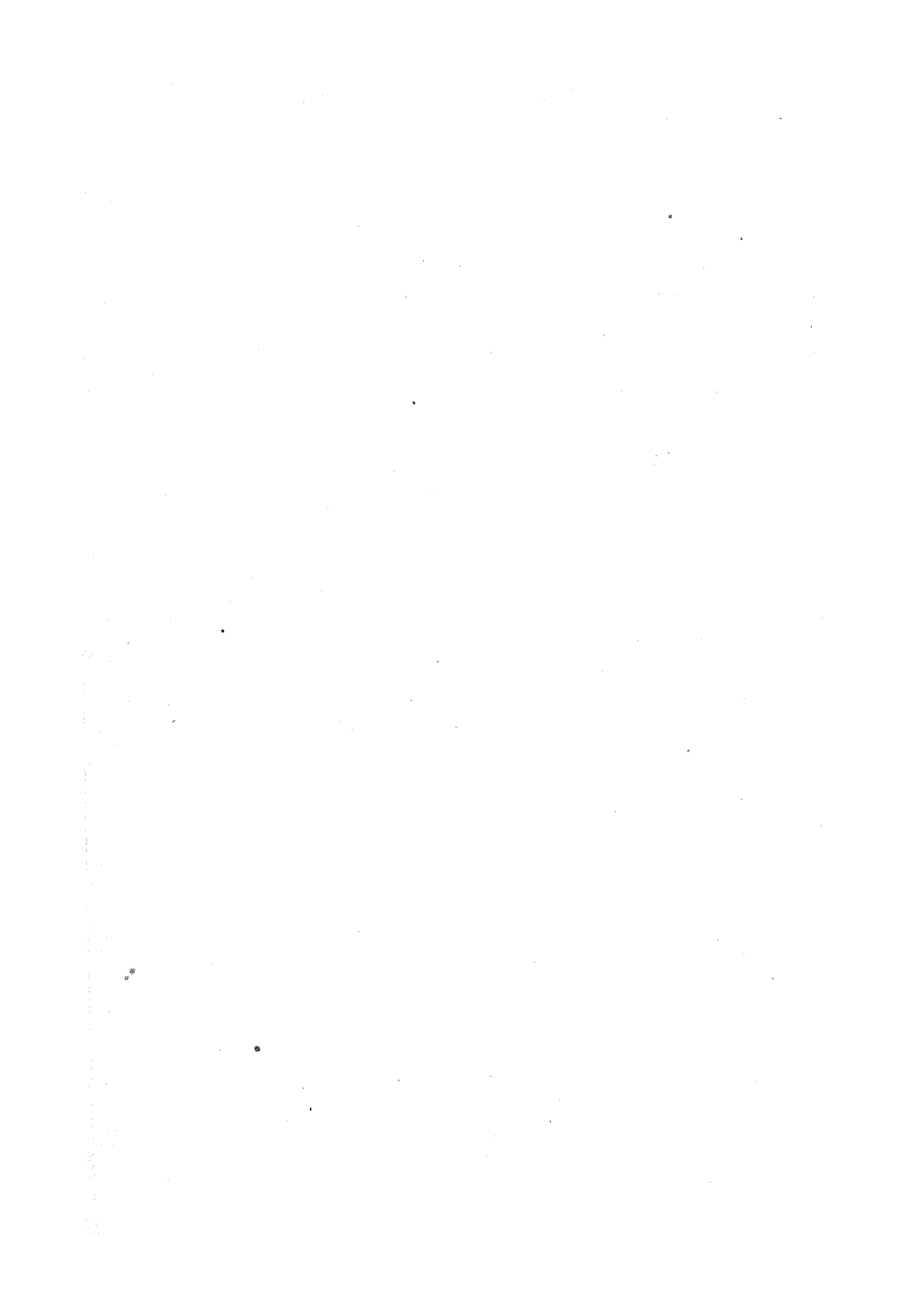
Changes in basal media, in method of sterilization, and in inocula produced no significant effects. A pH of 6.8 was more favorable to utilization of the methyl ester than pH 5.6. High levels of biotin methyl ester gave a greater percentage activity than low levels. Longer incubation periods increased percentage activity, although complete utilization of the methyl ester at low levels was not reached. Under the various conditions studied, the activity of the methyl ester ranged from 0 to 100%. Such variations make imperative the use of the biotin free acid, rather than the methyl ester in assays with *L. casei*.

Other factors affecting the biotin assay were studied. Variations in standard curves were shown to be due in part to variations in the casein hydrolyzate of the media. Copper salts in low concentration inhibited acid production. Replacement of yeast filtrate by vitamin B₆ resulted in a lag in acid production in 3-day incubations.

A biotin assay in a medium of known composition is possible if the incubation period is lengthened to 4 days. However, a synthetic medium is probably less satisfactory for assay purposes than one containing yeast filtrate. The latter appears to contain unknown substances that are slightly stimulatory. Such substances may also be present in the sample under assay, and their effect can be obviated by inclusion of yeast filtrate in the basal medium.

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Studies on Clot Strength of Dehydrated Plasma*

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INTRODUCTION

Since the use of freshly prepared plasma for the suture of peripheral nerves has given encouraging results (1, 2, 3), it has seemed worthwhile to determine whether clots formed from dehydrated plasma might be used for this purpose. Breakage strength is one of the main criteria for determining the suitability of plasma clots as suture material, and it was therefore considered necessary to investigate this property of clots prepared from desiccated plasma. This study is also concerned with the effect of various agents and conditions on the strength of clots prepared from dehydrated plasma.

EXPERIMENTAL

The blood for all experiments was obtained from dogs anesthetized with nembutal. It was drawn through a cannula lined with mineral oil which was inserted into the carotid artery. For the preparation of unmodified plasma (plasma to which no anti-coagulant is added), the blood was received into chilled paraffin-lined test tubes. The tubes were placed into 250 cc. metal cups filled with ice and were centrifuged for from 5 to 7 minutes at 2750 r.p.m. After removal from the centrifuge the plasma was then transferred to a paraffin-lined flask chilled in ice and pooled with other portions of plasma prepared in the same way.

The method selected for dehydrating plasma is that of vacuum drying in the frozen state, as recommended by Flosdorf and Mudd (4) and Link, Eggers, and Moulton (5) for the preparation of thermo-labile biologically active proteins. The apparatus used was that described by Hays and Koch (6). An ethyl alcohol bath chilled by solid carbon dioxide was used to freeze the pooled plasma (in 60-125 cc.

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portions) to be evaporated, while methyl cello-solve containing solid carbon dioxide was placed in the vacuum bottles of the dehydration apparatus.

Clot strengths were determined according to the method previously described (7) and subsequently modified (8) except that the clots, all of which were formed from 1 cc. of plasma, were not dried with filter paper before testing. Each breakage strength recorded in Tables 1, 3, and 4 is the average of six determinations. All other clot strengths represent an average of 3-6 determinations, depending on the amount of plasma available. Clot strength measurements were made 30 minutes after the "tip test" was positive since it has been shown (9) that after this time there is hardly any change in the breakage strength of clots.

Dehydration of Unmodified Plasma

Flosdorf and Mudd (4) and Strumia (10) observed that the pH of distilled water solutions of dried citrated plasma was higher (pH 8.8-9.2) than that of the original undehydrated citrated plasma (pH 7.4-7.5) and suggested that in the process of drying, carbon dioxide was removed. In their study of the stability of the prothrombin of lyophilized citrated human plasma, Kazal and Arnow (11) used dilute hydrochloric acid for pH restoration and found no apparent loss in prothrombin. In the present investigation, we also added enough dilute hydrochloric acid to the solution of dehydrated whole plasma in distilled water to bring the pH to 7.4-7.5.

A typical experiment for such a determination may be described as follows: 400 mg. dehydrated plasma + 3.4 cc. H_2O + 0.6 cc. 0.1 *N* HCl resulted in a solution with a pH of about 7.4, as determined with a glass electrode assembly (Beckman pH Meter).

The effect of dehydration upon the strength of clots prepared from unmodified plasma obtained from twenty different dogs is presented in Table I. It will be seen that there was almost invariably a loss of strength as a result of dehydration, the average loss being about 62 per cent.

Freezing of Plasma

In their studies on the preservation of citrated plasma, Strumia and McGraw (12) showed that preservation in the frozen state prevents bacterial growth and flocculation of fibrinogen and at the same time insures almost complete preservation of all specific elements including prothrombin and complement. They emphasized that thawing must be rapid and should, therefore, be carried out in the water bath at

TABLE I

Effect of Dehydration on Clot Strength of Dog Unmodified Plasma

Experiment No.	Clot Strength Before Dehydration	Clot Strength After Dehydration	Per cent Loss in Strength on Dehydration
1	22	11	50
2	75	19	75
3	46	48	0
4	114	85	25
5	63	15	76
6	20	<10*	>50
7	84	38	55
8	55	10	82
9	40	21	47
10	102	71	30
11	78	11	86
12	75	14	81
13	64	21	67
14	62	<10*	>84
15	81	14	83
16	78	36	54
17	55	<10*	>82
18	35	14	60
19	82	23	72
20	78	12	85

* Clot broke when the clamp containing paper cup was attached. Weight of clamp + paper cup = 10 grams. The clot strength in this and subsequent tables is given in grams.

37°C. This method seemed to offer another possibility for the preservation of the clot strength of unmodified plasma. Accordingly, 15 different specimens of unmodified dog plasma were frozen in paraffin-lined test-tubes at about - 8°C. using an ice-salt mixture, and were kept at that temperature for 4 hours (about the same length of time required for the dehydration of plasma in the experiments described above). These experiments were difficult to carry out because of the rather sudden clotting which often took place on thawing. However, a sufficient number of satisfactory determinations could be made to permit some conclusions to be drawn. The results were very similar to those obtained with the reconstituted dehydrated plasma, the average loss in strength being about 74 per cent. A second set of experiments using ten different samples of unmodified human plasma

instead of dog plasma gave similar results, the average loss in clot strength in these experiments being about 45 per cent (Table II).

TABLE II
Effect of Freezing on Clot Strength of Human Unmodified Plasma

Experiment No.	Clot Strength Before Dehydration	Clot Strength After Dehydration	Per cent Loss in Strength on Dehydration
1	78	56	28
2	69	35	49
3	79	82	0
4	66	65	1.5
5	24	<10	>58
6	77	33	57
7	74	41	45
8	74	21	72
9	34	<10	>71
10	28	<10	>64

Other Agents and Conditions Affecting the Clot Strength of Dehydrated Plasma

Many of the various conditions and agents which affect the tensile strength of clots prepared from fresh fluid unmodified plasma (8) act in a somewhat similar way on the strength of clots prepared from desiccated plasma except that the values in the latter case are in general lower.

1. Oxalated Plasma

For the dehydration experiments on oxalated plasma, the blood was oxalated according to the optimum conditions described previously (8). A 1.6% solution of a mixture of 1 part potassium oxalate and 1.5 parts of ammonium oxalate was prepared. One cc. of this solution was added to 19 cc. of whole blood. Solutions of the dehydrated oxalated plasma were made up as follows: To 400 mg. of dehydrated oxalated plasma in 3.3 cc. water were added 0.3 cc. 0.1 N HCl and 0.4 cc. 2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, giving a pH of 7.3 to 7.4. Measurements of strength were made on clots prepared from the undehydrated oxalated plasma by adding to 0.9 cc. of the plasma 0.1 cc. of 2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (pH 7.4 approximately). The results are shown in Table III.

It will be seen that the average per cent loss of strength on dehydration of oxalated plasma was 44.

TABLE III

Effect of Dehydration on Clot Strength of Dog Oxalated Plasma

Experiment No.	Clot Strength Before Dehydration	Clot Strength After Dehydration	Per cent Loss in Strength on Dehydration
1	31	<10	>68
2	39	28	28
3	16	<10	>38
4	25	20	20
5	31	16	48
6	32	17	47
7	71	47	34
8	<10	<10	*
9	25	11	56
10	<10	<10	*
11	48	21	56
12	23	13	43
13	12	<10	*

* Value could not be determined.

2. Citrated Plasma

Blood was citrated by adding to 18 cc. of it 2 cc. of 3.8% $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 5\frac{1}{2}\text{H}_2\text{O}$. For each experiment about 100 cc. of the plasma was dehydrated from the frozen state. Solutions were prepared as follows: To 400 mg. of dehydrated citrated plasma in 3.2 cc. H_2O were added 0.4 cc. 0.1 N HCl + 0.4 cc. of 2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, giving a solution with a pH of 7.3 to 7.4. For clot strength determinations of the original citrated plasma 0.1 cc. of 4% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were added to 0.9 cc. plasma. The results are shown in Table IV.

The average loss of clot strength of citrated plasma was found to be 51%.

3. The Effect of Concentration of Unmodified Plasma on Clot Strength

It also appeared of interest to study the effect of variations of concentration of dehydrated whole plasma solutions on the clot strength. If the clot strength of solutions containing dehydrated plasma could be increased merely by using larger quantities of the dehydrated material, this might constitute a decided advantage for the application of clots prepared from dehydrated plasma for suturing nerves. Table V shows the results using five different preparations of

TABLE IV
Effect of Dehydration on Clot Strength of Citrated Plasma

Experiment No.	Clot Strength Before Dehydration	Clot Strength After Dehydration	Per cent Loss in Strength on Dehydration
1	99	93	6
2	15	10	33
3	56	10	82
4	41	30	27
5	35	12	66
6	32	11	66
7	16	12	25
8	24	10	58
9	57	13	77
10	59	10	83
11	14	10	29
12	16	<10	*
13	27	12	56

* No figure can be given.

TABLE V
Relationship Between Concentration of Dehydrated Plasma Solutions and Clot Strength

Preparation No.	Clot Strength of Solutions					
	3.8%	7.4%	9.1%	10.7%	13.8%	16.7%
1	<10	14	13	<10	31	42
2	16	36	33	33	39	64
3	<10	<10	<10	27	43	<10
4	<10	14	13	16	21	—
5	<10	19	23	24	18	<10

dog plasma. Each aliquot was brought to the same pH range (7.3–7.4) with 0.1 N HCl, and the concentrations are as listed in the table.

It was found in general that the 3.8% solutions by weight gave the weakest clots, while stronger clots were obtained in the range of 7.4 to 13.8% with somewhat better results in 4 of the 5 preparations containing 13.8% dehydrated plasma. It was more difficult to work with the 16.7% concentrations due to the longer time required for solution, their higher viscosity, and the prolonged clotting time (in some instances one hour compared with an original clotting time of 150 to 180 seconds over the range of 7.4 to 13.8% solutions). In no case was the strength of the clots prepared from the original liquid plasma attained.

4. *The Influence of the Method and Duration of Storage on Clot Strength*

Some ampoules containing dehydrated whole plasma were evacuated and sealed, others were filled with carbon dioxide at atmospheric pressure and sealed, and others were sealed in air. Preparations were selected for these experiments which had a high clot strength after dehydration.

One preparation stored in air at room temperature dropped in clot strength from an initial value of 63 to less than 10 after 23 days, while when kept in carbon dioxide in the refrigerator, the clot strength was 53 after 23 days. Another preparation had an initial clot strength of 114 and when stored in a sealed ampoule, containing carbon dioxide, at room temperature gave a clot strength of 29 g. after 21 days. However, when one aliquot was stored in carbon dioxide and another in air in the refrigerator, the clot strengths were 81 and 80 respectively after 21 days. After 45 days when kept at 4°C. and sealed in carbon dioxide, the clot strength had dropped to 47, while an aliquot sealed in vacuo and kept at 4°C. for the same length of time gave a clot strength of 46. Thus the temperature appears to be more important than the medium of storage in preventing deterioration in strength of clots prepared from dehydrated plasma.

5. *Clotting Time, Clot Strength, and pH of Unmodified Plasma*

It should be mentioned at this point that solutions of dehydrated whole plasma were found to clot readily within the pH range of 6.9 to 7.5. (Outside of this range, clot strength was found to decrease considerably with change in pH, there being practically no clot formation below pH 6.4 and above pH 8.3 for the 9.1% solutions by weight.) No great variations in clotting time and clot strength were found over this pH range except at a pH of 7.5 as is illustrated in Table VI, which represents a typical experiment for a 9.1% solution by weight of dehydrated whole plasma.

TABLE VI
Clot Strength and Clotting Time at Different pH

pH	Clot Strength	Clotting Time (in secs.)
6.8	20	195
7.0	17	210
7.3	27	220
7.5	16	122

The question arises as to whether there is any correlation between clotting time and clot strengths of different dehydrated plasma preparations. An examination of data obtained from many different preparations of unmodified plasma solutions reveals no significant relationship between these properties. The following table shows two such sets of experiments as an illustration of this point, and many more could easily be cited:

TABLE VII
Clot Strength and Clotting Time of Different Preparations

Date of Preparation	pH	Clotting Time (secs.)	Clot Strength
3/26/43	7.2	160	<8
5/14/43	7.4	165	63
4/ 9/43	7.4	90	88
5/22/43	7.4	90	19

DISCUSSION

The experiments reported here point to the conclusion that the clot strength of plasma, whether unmodified, citrated or oxalated, is markedly decreased as a result of dehydration. The results raise the interesting question regarding the factor or factors involved in loss of clot strength, which follows the dehydration of plasma. While in general it is believed that most constituents of the plasma are preserved during the high-vacuum-low-temperature-drying technique, a few are reported to undergo some change. Desiccation of plasma produces an apparent alteration in the albumin-globulin-ratio as determined by the usual laboratory methods, but no indication of the alteration of the proteins is found in the electrophoretic pattern obtained by the Tiselius apparatus (12, 13). According to Strumia (12), the complement factor is not always preserved. Whether the decreased breaking strength of clots in restored plasma observed in this study is related to one of the above factors or is associated with a change in a hitherto unobserved factor requires further investigation.

CONCLUSIONS AND SUMMARY

1. Vacuum drying of plasma in the frozen state resulted in a loss of roughly about one-half the tensile strength of clots prepared from oxalated, citrated, or unmodified plasma.

2. Optimum clotting of solutions of reconstituted dehydrated plasma occurs in the pH range of 6.8 to 7.4.

3. Clots prepared from thawed frozen plasma also exhibit a loss of strength of one-half or more.

4. Of the solutions of dehydrated plasma tested (3.8% to 16.7%), stronger clots were obtained from those solutions within the range 7.4%–13.8% (pH 7.3 to 7.4).

5. The loss in clot strength was less when dehydrated plasma was stored at 4°C. than at room temperature.

6. No correlation was found between clotting time and clot strength of different preparations.

It is a pleasure to again thank Dr. A. S. Wiener for his most helpful interest in this study.

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Studies on the Pigments of the Purple Bacteria

II. A Spectroscopic and Stereochemical Investigation of Spirilloxanthin

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An intensely purple-red carotenoid, the well-crystallized spirilloxanthin (Fig. 1), has previously been described by van Niel and Smith

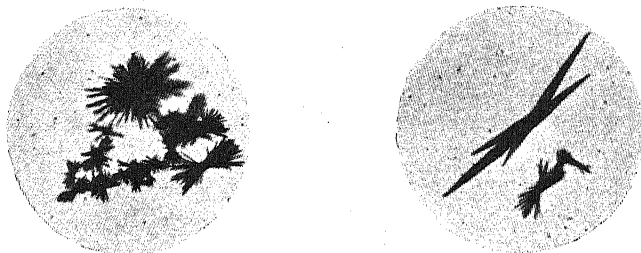


FIG. 1

All-*trans*-spirilloxanthin, Crystallized from Carbon Disulfide and Petroleum Ether (200 \times)

(8) as the predominant polyene pigment of the non-sulfur purple bacterium, *Rhodospirillum* (*Spirillum*) *rubrum* (Family *Athiorhodaceae* Molisch (7)). At that time the presence of a number of other carotenoids in the same microorganism was mentioned but those polyenes were characterized only by some spectral and adsorption data. A re-investigation has now shown that the majority do not occur in fresh cultures. Thus, they very probably are alteration products of spirilloxanthin which accounts for at least 95% of the native carotenoid pigment in *Rhodospirillum rubrum*.

A pigment with characteristics very similar to those of spirilloxanthin, and designated as rhodoviolascins, was subsequently isolated by Karrer and Solmssen (3) from mass cultures of non-sulfur purple bacteria, identified as *Rhodovibrio*. Rhodoviolascins were here found accompanied by several other carotenoids, some of which occurred in considerable amounts. Karrer and Solmssen concluded from their observations that *Rhodovibrio* is capable of synthesizing a variety of polyene pigments, and that the proportion in which these are formed is a function of the environmental conditions under which the organism is cultured. Nevertheless, it must be remarked that these mass cultures of *Rhodovibrio* did not by any means represent pure cultures, but contained a mixture of different bacterial species, including various members of the *Athiorhodaceae*. Hence the actual source of rhodoviolascins is somewhat in doubt, and it is conceivable that this pigment was isolated as a result of the presence, in the mass cultures, of *Rhodospirillum rubrum* in addition to *Rhodovibrio* and other species of non-sulfur purple bacteria, the latter having been responsible for the formation of the other carotenoids. Karrer and Koenig (4) later encountered rhodoviolascins as the only polyene pigment in "red mud" from Eastern Africa. Mass developments of purple bacteria in nature have, without exception, been traced to the group of sulfur purple bacteria (Family *Thiorhodaceae* Molisch), as particularly stressed by Buder (1). It thus appears that the pigmentation of the last-mentioned organisms is quite comparable to that of *Rhodospirillum rubrum*. A detailed discussion of the present status of our knowledge of the pigments of the purple bacteria can be found elsewhere (9).

There are good reasons for admitting the identity of spirilloxanthin and rhodoviolascins. Earlier reports of the absorption characteristics and melting points of the two pigments were strongly indicative in this respect. It is true that the formula, $C_{48}H_{66}O_3$, originally proposed (8) with reservations for spirilloxanthin, differed markedly from that established by Karrer and Solmssen (3) for rhodoviolascins as $C_{40}H_{54}(OCH_3)_2$. However, since that time the presence of two methoxyl groups in the spirilloxanthin molecule has been ascertained (van Niel and Wiedemann, unpublished; cf. our Experimental Part), while also the new analyses reported below are in good agreement with the formulation of Karrer and Solmssen. Of decisive importance is the determination of the molecular weight of perhydro-spirilloxanthin. While, as a result of present circumstances, a final identification of

spirilloxanthin and rhodoviolascins must be postponed, we believe that there are no valid reasons for doubting their identity, and now adopt Karrer and Solmssen's formula also for the former pigment as well as the presence of thirteen double bonds.

The small differences in the visually established position of the absorption maxima of spirilloxanthin and rhodoviolascins respectively in various solvents (Table I) do not seem to be significant. They can

TABLE I

Visually Observed Spectral Maxima of Spirilloxanthin in Different Solvents Compared with Those of Rhodoviolascins (Karrer and Solmssen) ($m\mu$)

Solvent	Rhodoviolascins			Spirilloxanthin			Spirilloxanthin + iodine	
Carbon disulfide	573.5	534	496	571.5	532	495	569.5	530
Benzene	548	511	482	548.5	510	479	543	505
Carbon tetrachloride		—		545.5	507	(475)	543.5	503.5
Chloroform	544	507	476	543	505	(475)	542.5	504
Dioxane		—		540.5	503.5	473	537	500.5
Petroleum ether (b.p. 60–70°)		—		528	493	461	524.5	490

readily be ascribed to differences in instruments and, especially, to the extreme steric lability of the pigment. It has been found that within two hours the position of the absorption maxima of a fresh spirilloxanthin solution in benzene, at room temperature, shifts 1 to 2 $m\mu$ towards shorter wave lengths. The position of the extinction maxima and minima of spirilloxanthin and rhodoviolascins, the latter determined by Karrer and Würgler (5), are also well comparable (Table II). This is not the case for the respective molecular extinction coefficients; the reason for the discrepancy will be discussed in the Experimental Part.

Spirilloxanthin has no provitamin A activity in the rat when applied in daily doses of 60 μg .

In the present paper we intend to give the first stereochemical discussion of a bacterial polyene pigment.

During the last decade steric changes of the most important C_{40} carotenoids of higher plants have been investigated repeatedly. The pioneer observation in this field was made by Gillam and El Ridi (2) and early contributions were made by Strain (12) and by other authors. The history and the present state of the stereochemistry of polyenes have been recently outlined in a review (14).

TABLE II

Comparison of the Positions of the Maxima (*italicized*) and Minima for Rhodoviolascin (Karrer and Würgler) and Spirilloxanthin in Hexane ($m\mu$)

Rhodoviolascin (instrument not specified)	Spirilloxanthin (Beckman spectrophotometer)
<i>307</i>	<i>305</i>
<i>310</i>	<i>310</i>
<i>318</i>	<i>317</i>
<i>340</i>	<i>338-339</i>
<i>370</i>	<i>368-369</i>
<i>375</i>	<i>376</i>
<i>386</i>	<i>386</i>
<i>400</i>	<i>396</i>
<i>461</i>	<i>465</i>
<i>473</i>	<i>475</i>
<i>493</i>	<i>493</i>
<i>511</i>	<i>511</i>
<i>525</i>	<i>527</i>

It was shown by one of the authors in collaboration with Chohnoky, Tuzson, and Polgár (15) that the common carotenoids which possess an all-*trans* configuration easily undergo a reversible *trans*→*cis* rearrangement when kept or heated in solution or when catalyzed with iodine. A partial isomerization occurs also upon the melting of crystals, under the influence of acids (12), or exposure of solutions to sunshine (20). In each case a more or less complicated mixture of stereoisomers is formed in which ordinarily the unchanged portion of the all-*trans* compound predominates. The components of such a mixture can be conveniently separated on a Tswett column.

Partially *cis* carotenoids of the C_{40} class also occur in nature, and two such pigments, polycopene, $C_{40}H_{56}$, and pro- γ -carotene, $C_{40}H_{56}$, were isolated recently, in collaboration with LeRosen, Schroeder, and Escue (16, 17).

According to Pauling (10, 17), steric hindrance will not permit certain double bonds of a carotenoid molecule to undergo *trans*→*cis* rotations.

When spirilloxanthin was investigated with the above principles in mind, the following results were obtained.

This bacterial pigment is also subject to reversible *trans*→*cis* rearrangements. The main product of the spatial alteration is an orange-red pigment which is adsorbed on the chromatographic column immediately below the unchanged portion of the starting material and

which has been termed neospirilloxanthin A. The lowest sections of this zone contain, to a variable extent, some poorly separated minor isomers among which a neo B has been differentiated. The spectral maxima of these isomers are located at shorter wave lengths than those of natural spirilloxanthin. After iodine catalysis and illumination of either member of the stereoisomeric set the spectral maxima lie between those of natural spirilloxanthin and its neo-forms mentioned in Table III.

TABLE III
Visually Observed Spectral Maxima of Some Members of the Stereoisomeric Spirilloxanthin Set ($m\mu$)

Member of the set	In benzene			In petroleum ether (b.p. 60-70°)		
All- <i>trans</i>	548.5	510	479	528	493	461
Neo A	537.5	499.5	466.5	518	486	454.5
Neo B	528.5	493	461	511.5	479.5	449.5
Mixture obtained upon iodine catalysis	543	505	(474)	524.5	490	(458)

The unusual lability of the all-*trans* configuration makes stereochemical work on spirilloxanthin solutions difficult. The extent of spontaneous isomerization within a day at room temperature is only 1 to 2% for α -carotene, β -carotene, cryptoxanthin, or capsanthin, and 10% for lycopene (14), but it amounts to 23% for spirilloxanthin. Chromatography shows that even "fresh" solutions contain appreciable amounts of isomerized pigment.

Upon iodine catalysis spirilloxanthin in benzene yields very rapidly a mixture in which the colorimetric ratio, unchanged all-*trans* : neo-forms is about 55 : 45. The elution of that layer which contains the main isomer formed (neo A) and transfer into benzene is sufficient to re-establish the equilibrium just mentioned. A similar result was obtained when all operations were carried out between 5° and 10° in darkness within 9 minutes. Sterically homogeneous neospirilloxanthin A can, therefore, be obtained as adsorbate only, not as a solute.

When natural spirilloxanthin is melted with naphthalene, rapid stereoisomerization and partial cleavage take place. The main product of the second process is a well characterized light-yellow pigment which will be investigated later.

Exposure of spirilloxanthin solutions to sunshine also increases the rate of the spontaneous *trans*→*cis* rotations.

We may mention that any stereochemical change alters the state of the conjugated system and hence the spectrum. It has been shown that among all members of a stereochemical set the all-*trans* compound must have the longest wave length spectral maxima (11).

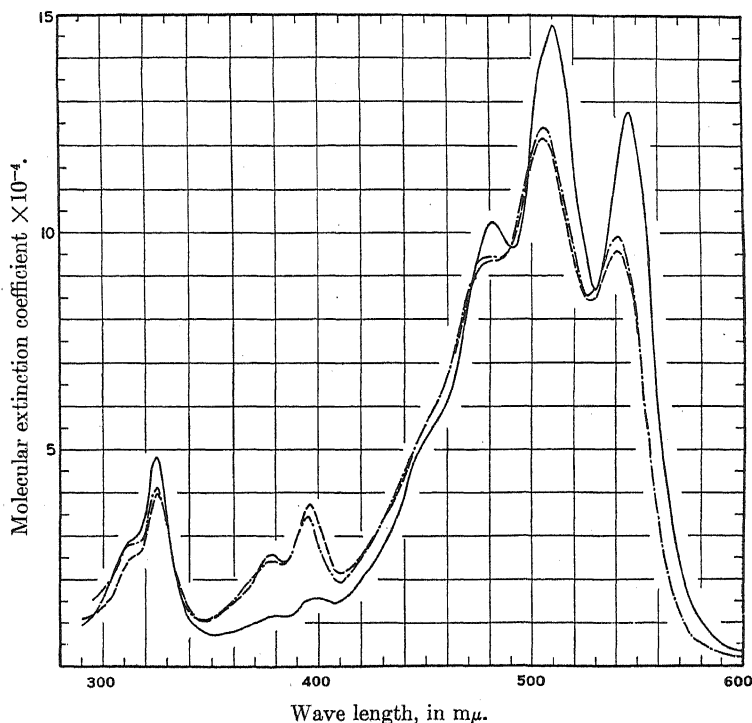


FIG. 2

Molecular Extinction Curves of Spirilloxanthin in Benzene

- , fresh solution of the all-*trans* compound.
- - -, mixture of stereoisomers after refluxing in darkness for forty-five minutes.
- · - ·, after iodine catalysis at room temperature in light.

While upon stereoisomerization of an all-*trans* carotenoid the height of its extinction maxima decreases in the visible region, it was shown by Zechmeister and Polgár (18) that such a bending of the molecule brings about a new maximum, the so-called "*cis*-peak," which is

located in the near ultra-violet region, somewhere between 320 and 380 $m\mu$. The *cis*-peak effect was given a theoretical interpretation in collaboration with Pauling, LeRosen, and Schroeder (19).

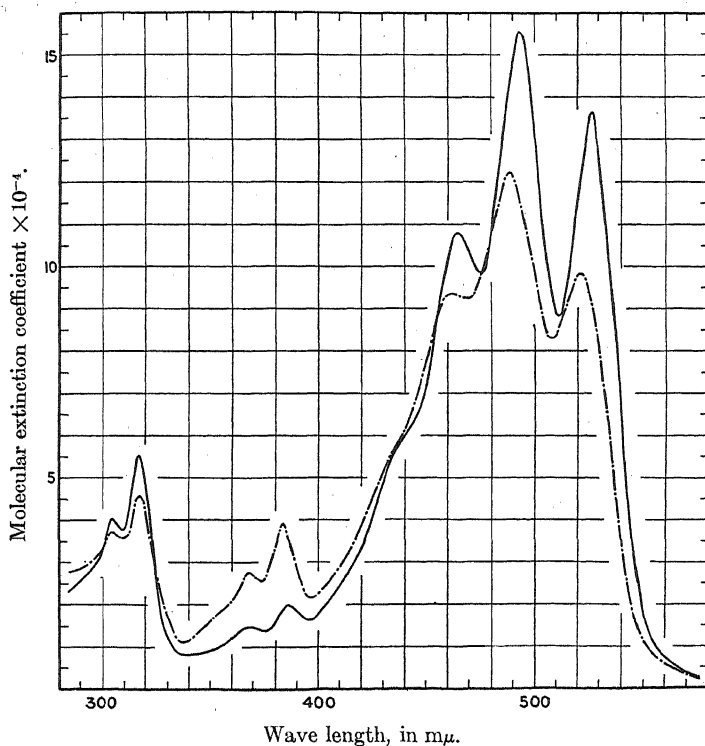


FIG. 3

Molecular Extinction Curves of Spirilloxanthin in Hexane

—, fresh solution of the all-*trans* compound.

- - -, mixture of stereoisomers after iodine catalysis at room temperature in light.

In Figs. 2 and 3 we present the extinction curves of all-*trans* and isomerized spirilloxanthin in benzene and hexane solution. The position of the sharp *cis*-peaks is 394-5 $m\mu$ and 384 $m\mu$ respectively. A curve for natural spirilloxanthin in carbon disulfide solution was tentatively published earlier (8) and was calculated on the basis of a higher molecular weight (690 instead of 597). It is now replaced by

Fig. 4. The fundamental band in carbon disulfide shows a somewhat less fine structure than in benzene or hexane; furthermore, iodine produces no steep *cis*-peak but a general elevation of the all-*trans* curve in the *cis*-peak region. No noticeable fine structure appears although

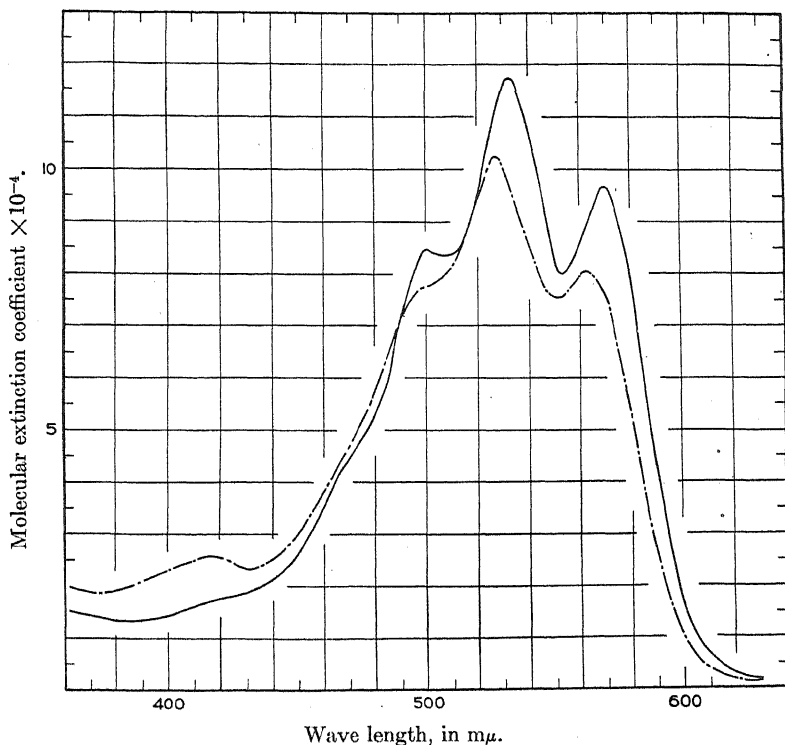


FIG. 4

Molecular Extinction Curves of Spirilloxanthin in Carbon Disulfide

—, fresh solution of the all-*trans* compound.

- - -, mixture of stereoisomers after iodine catalysis at room temperature in light.

the usual stereoisomers are present as was proved by chromatography. It was briefly mentioned elsewhere that lycopene shows a similar behavior (14). When increasing amounts of carbon disulfide are added to an iodine-catalyzed benzene solution of spirilloxanthin, a gradual flattening of the molecular extinction curve takes place in the region

of the *cis*-peak, the position of which simultaneously shifts toward longer wave lengths (Fig. 5).

On the basis of the available observations spirilloxanthin as produced by the purple bacteria must possess all-*trans* configuration.

The only other member of this stereoisomeric set whose configuration can reasonably be discussed at the present time is the major isomer, neospirilloxanthin A. As shown by the unusually rapid spontaneous isomerization of natural spirilloxanthin, neo A is formed with

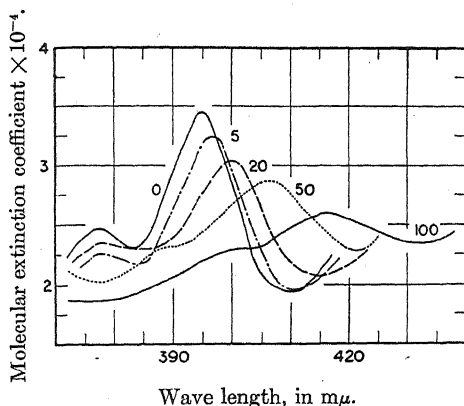


FIG. 5

Alteration of the Molecular Extinction Coefficient in the *cis*-Peak Region of an Iodine-catalyzed Spirilloxanthin Solution in Benzene, upon the Addition of Carbon Disulfide

(The figures on the curves indicate per cent carbon disulfide in the solution.)

very great ease. Furthermore, according to Fig. 6, it possesses a markedly higher *cis*-peak than the iodine-catalyzed equilibrium mixture in which it occurs to an extent of about 40%. The value of its molecular extinction coefficient at the *cis*-peak wave length is 5.1×10^4 in hexane. It should be noted that this represents a minimum value. Indirect calculation (on the basis of the *cis*-peak of the all-*trans* form and of the equilibrium mixtures of known composition) gave about $E_{1\text{ cm.}}^{\text{mol.}} = 6 \times 10^4$. Indeed, in a single, unreproducible experiment the value 7×10^4 was found (in alcohol). It has previously been explained in detail (19) that in the chromophoric system of carotenoids such high *cis*-peaks indicate the presence of a centrally located *cis*-double bond.

In the field of the common carotenoids occurring in higher plants it was found regularly that the visually established spectral difference between the longest wave length maximum of an *all-trans* pigment and any of its mono-*cis* isomers investigated is about 5 $m\mu$ in petroleum

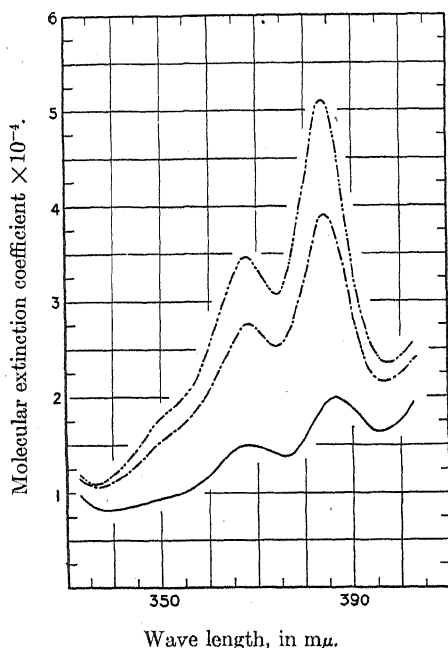


FIG. 6

Molecular Extinction Curves of *All-trans*-spirilloxanthin and Neospirilloxanthin A in the *cis*-Peak Region in Benzene

- , fresh solution of the *all-trans* compound.
- · - ·, fresh solution of neospirilloxanthin A.
- - -, mixture of stereoisomers after iodine catalysis of the *all-trans* compound at room temperature in light.

ether (19). We believe that this result may be applied to a pigment of the spirilloxanthin type, and that neospirilloxanthin A ($\Delta = 10 m\mu$) must be assigned two *cis* double bonds, one of which has a central location in the chromophore.

EXPERIMENTAL

I. Preparation and Analysis of Spirilloxanthin

The pigment samples were prepared in the manner previously described (8). The starting material consisted of dried *Rhodospirillum rubrum* cells, collected by centrifugation from pure cultures of this species. It was ground to a fine powder from which the carotenoid pigment was extracted with carbon disulfide, and isolated by crystallization from carbon disulfide-petroleum ether. A number of other methods have now been tested, including the simultaneous extraction with two non-miscible solvents, recommended by Went, LeRosen, and Zechmeister (13), but none has been found quite as satisfactory as the original procedure.

When fresh cultures are available, the preliminary drying can profitably be replaced by a direct treatment of the cell suspension with seven volumes of methanol. This step not only serves to dehydrate the bacteria but also accomplishes an almost quantitative extraction of the bacteriochlorophyll while practically the entire carotenoid content remains in the cells. The latter can be separated readily with the aid of a centrifuge but not by filtration. After the residue has been washed with a small amount of methanol, it is exhaustively extracted with carbon disulfide or benzene.

The use of acetone instead of methanol for the initial dehydration causes the bacterial cells to clump together so that they can subsequently be filtered off without difficulty. This slight advantage is, however, offset by the fact that the acetone solution contains a considerable amount of the red pigments, while only incomplete elimination of the bacteriochlorophyll is accomplished. Furthermore, continued treatment with acetone does not result in a quantitative extraction of spirilloxanthin. The solubility characteristics of the latter are such that only carbon disulfide, benzene, and chloroform appear to be useful as solvents.

Chromatographic analysis of the carbon disulfide or benzene extracts of fresh cultures on calcium hydroxide (or magnesium oxide) columns shows them to contain a small amount of bacteriochlorophyll, which is adsorbed in the uppermost layer. When the red zone immediately below is developed with either of the two solvents, it separates into two zones. The upper one, which is by far the larger, contains spirilloxanthin; the lower one contains orange-colored neospirilloxanthins. During the development a yellow pigment, present in minute quantity, is washed through the column; it shows no maxima above $350\text{ m}\mu$ (in benzene).

The crystalline spirilloxanthin samples resulting from the extraction of the dried bacteria were further purified by chromatography (cf. Section II). The eluted spirilloxanthin was transferred into benzene and, after complete evaporation of the solvent in vacuo, dissolved in the necessary volume of chloroform at 25° . Upon the addition of an excess of light petroleum ether (b.p. $28\text{--}38^\circ$), the violet-red platelets appeared almost immediately. They were kept for some time at 5° , filtered, washed with cold petroleum ether, and dried at 56° under 0.1 mm. pressure for an hour.

Analysis. The two first estimations were carried out with an independent sample. Even our purest substances contained 0.8% ash for which the values are corrected.

$\text{C}_{40}\text{H}_{54}(\text{OCH}_3)_2$. Calculated C	84.48,	H 10.16,	OCH_3 10.40.
Found	84.50, 84.71, 83.86,	10.26, 10.06, 10.60,	9.75.

Karrer and Solmssen (3) determined for rhodoviolascin, 84.49% C; 10.39% H; and 10.04% OCH_3 .

Catalytic hydrogenation. Karrer and Solmssen (3) found 13.5 and 12.85 double bonds in rhodoviolascin. The corresponding value published by van Niel and Smith (8) is 12.8 when calculated on the basis of the accepted molecular weight (597).

The perhydrogenated spirilloxanthin now obtained formed a viscous, practically colorless oil with a faint, terpene-like smell. It was dried in a thin layer with occasional stirring in high vacuum at 56° for one hour and a half.

Molecular weight of perhydro-spirilloxanthin. 0.518 mg. substance in 4.040 mg. camphor ($K = 36.4$): $\Delta = 7.5^\circ$; 0.491 mg. substance in 2.184 mg. exaltone ($K = 21.3$): $\Delta = 7.6^\circ$.

$\text{C}_{42}\text{H}_{86}\text{O}_2$. Calculated MW 623.

Found 622, 630.

This experiment replaced an estimation with native spirilloxanthin, the solubility of which is unsatisfactory. (For the same reason no Zerewitinoff test, either in pyridine or in anisole, could be carried out.)

The above values show conclusively that spirilloxanthin belongs to the C_{40} -carotenoids. The extraordinarily good checking between calculated and found molecular weights is, however, a mere coincidence since our perhydrogenated crude sample did not give satisfactory carbon and hydrogen values.

II. Materials and General Methods

The "petroleum ether" used was Skellysolve B, b.p. $60-70^\circ$. Hexane (Eastman Kodak Co., from petroleum, practical) was purified with fuming sulfuric acid, followed by treatment with alkaline permanganate and fractionation (b.p. $62-5^\circ$). A more convenient and effective purification of hexane by means of chromatographic filtration through silica gel was developed by Polgár (unpublished). Other solvents used include carbon disulfide (Reagent, Baker and Adamson), and benzene (Reagent, Baker and Adamson, "thiophene free"). For spectrophotometric experiments the benzene was purified by repeated treatments with fuming sulfuric acid and alcoholic potassium hydroxide.

A 2:1 mixture of calcium carbonate (Michigan Alkali Co.) and calcium hydroxide (Shell brand lime, chemical hydrate; 98% through 325 mesh¹) was used. The columns were developed with benzene containing 0.5% to 2% acetone or with petroleum ether containing 5 to 10% acetone. The best eluent for spirilloxanthin and its isomers is a mixture of alcohol and benzene 1:1; alcohol-petroleum ether mixtures do not elute quantitatively. After elution the alcohol was rapidly washed out in a continuous process (6). Carbon disulfide solutions were chromatographed on calcium carbonate (Merek, Heavy Powder), and the pigment was eluted with acetone + carbon disulfide. Refluxing experiments were carried out in darkness while a slow stream of nitrogen was introduced into the all-glass apparatus. The iodine catalyzed solutions were exposed to diffuse daylight in 25 ml. glass volu-

¹ Standardization, cf. *J. Am. Chem. Soc.* **66**, 140 (1944), Footnote 10 (suggested by Dr. A. L. LeRosen).

metric flasks on a white plate for 30 min. or, preferably, illuminated by two 3500° white Mazda fluorescent lamps (tube length, 120 cm.) at a distance of 60 cm. for 15 min.

The visual spectra were taken with an Evaluating Grating Spectroscope (Zeiss, light filter BG-7; 2 mm. thick). The spectral data refer to benzene solutions unless otherwise indicated. After observing the position of the maxima, one or two drops of iodine solution were added to the cell and the readings were repeated a few minutes later. This treatment shifted the visual maxima of any member of the spirilloxanthin set to 543, 505, 474 m μ in benzene. Fresh solutions with a concentration of 2.5 to 3.5 mg. pigment per liter (about 5×10^{-6} molar) were used to determine the extinction curves. When a portion of the solution was refluxed, the conditions were the same as those described below in Section IVa. The essential points of the curves were taken first. A molecular weight of 597 was used for the calculations. The molecular extinction coefficient is defined (18) by the expression $E_{1\text{ cm.}}^{\text{mol.}} = \frac{1}{L \cdot c} \log \frac{I_0}{I}$ where c = moles per liter.

III. Procedures and Calculations Connected with Extinction Measurements

(a) *Benzene solutions.* (Figs. 2, 7, 8; Tables IV, V.) The concentration of solutions used to obtain the molecular extinction curves of spirilloxanthin (Fig. 2) was determined by weighing. In other cases concentrations were computed by adding 1 or 2 drops of a strong iodine solution and, after illumination, measuring the extinction at 505 m μ . This value was then compared with the molecular extinction coefficient of the iodine equilibrium mixture at the same wave length, *viz.* $E_{1\text{ cm.}}^{\text{mol.}} = 12.5 \times 10^{-4}$ which was based on direct weighings.

The all-*trans* curve in Fig. 2 is the average of two experiments which differed in the heights of the maxima in the visible region by 1%. Because of the rapidity of spontaneous isomerization and the low rate of dissolution of the crystals, the *cis*-peak region of this curve was measured in separate experiments. For that purpose spirilloxanthin crystals were shaken with benzene for 1 to 2 minutes, and the suspension was filtered rapidly; the *cis*-peak could be measured four minutes after the crystals and solvent came into contact. The heights of the *cis*-peak in Table IV represent the average of the two lowest values obtained. Fig. 7 shows that the extinction at the *cis*-peak increases rapidly on standing.

In the iodine equilibrium curve (Fig. 2) the uncertainty amounts to 2 to 3% at the highest maximum.

(b) *Hexane solutions.* (Figs. 3, 6; Table VI.) Pure spirilloxanthin crystals are practically insoluble in cold hexane. Therefore, the samples were shaken with dioxane from which, regardless of a few floating platelets, the pigment (after filtering) was transferred with water into hexane. The solution was washed and dried. The first readings in the Beckman apparatus were made 8 to 9 minutes after the dioxane had been poured on the crystals. In order to determine the concentration, a drop or two of a strong iodine solution in hexane was added and after illumination the extinction was measured at the *cis*-peak wave length, *viz.* 384 m μ . The concentration (mg. per liter) is then calculated from the equation $c = 15.2 \times E_{1\text{ cm.}}$. This relation,

TABLE IV

Molecular Extinction Coefficients of Spirilloxanthin at the Maxima (italicized) and Minima in Benzene

Fresh solution		After heat isomerization		After iodine isomerization	
$m\mu$	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$	$m\mu$	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$	$m\mu$	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$
315 ^a	2.95	315 ^a	2.56	315 ^a	2.82
325	4.9	325	4.0	325	4.2
350	0.71	350	1.06	348	1.05
380	1.20	378	2.63	378	2.48
386	1.16	384	2.41	384	2.34
397	1.62	396	3.8	394-395	3.5
408	1.42	408	2.13	408-410	2.07
480-481	10.3	485 ^a	9.4	478-480	9.5
492	9.7			482-484	9.5
510	14.9	505	12.2	505	12.5
530	8.7	527-528	8.5	526	8.6
546	12.8	541	9.6	540	10.0

^a Point of inflection.

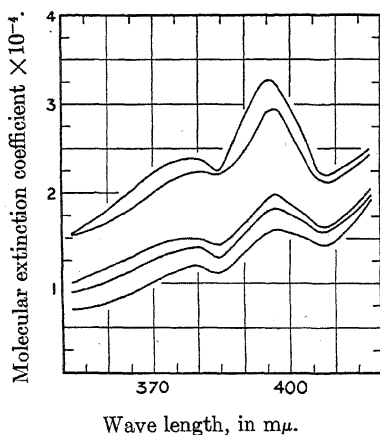


FIG. 7

Spontaneous Increase of the *cis*-Peak of Spirilloxanthin in Benzene

The curves from bottom to top represent the effect of 2½ min., 15 min., 30 min., 2½ hours, and 4 hours standing in darkness at room temperature after the dissolution of crystals

TABLE V

Influence of Illumination on the Development of the Cis-peak Effect in Benzene Solutions of Spirilloxanthin Catalyzed with Iodine

Duration of the illumination	Mol. extinction coeff. at the max. of longest wave length, $E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$	Mol. extinction coeff. at the wave length of the <i>cis</i> -peak, $E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$
0 sec.	13.6	2.12
5 sec.	12.8	3.42
30 sec.	12.4	3.48
2½ min.	12.4	3.49
15 min.	12.4	3.55
30 min.	12.4	3.48

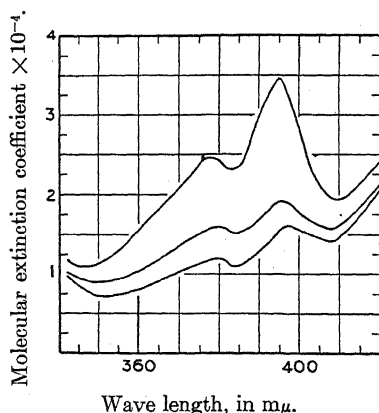


FIG. 8

Influence of Illumination on the Development of the cis-Peak Effect in an Iodine Catalyzed Solution of Spirilloxanthin in Benzene

Curves from bottom to top: after 0 sec. illumination without iodine; after 0 sec., and after 2½ min. (or 30 min.) illumination upon iodine catalysis

which is valid for hexane solutions, was found as follows. The pigment was transferred into 25 ml. of benzene and 25 ml. of hexane respectively from two 25 ml. portions of the same alcoholic solution (obtained by elution of a chromatographic zone). After iodine catalysis the extinctions of the benzene and hexane solutions were measured at 505 and 384 $m\mu$ respectively. The absolute concentration of the solutions could thus be determined from the value of the former with the aid of the molecular extinction coefficient for the iodine equilibrium mixture in benzene (12.5×10^4 at 505 $m\mu$). On the basis of the values so obtained the relationship between concentration and extinction in hexane at 384 $m\mu$ was established.

TABLE VI

Molecular Extinction Coefficients of Spirilloxanthin at the Maxima (italicized) and Minima in Hexane

Fresh solution		After iodine catalysis	
$m\mu$	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$	$m\mu$	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$
305	4.1	305	3.7
310	3.8	310	3.6
317	5.6	317-318	4.6
338-339	0.82	338	1.11
368-369	1.51	368	2.79
376	1.39	374	2.54
386	2.01	384	3.9
396	1.65	396	2.16
465	10.8	462	9.4
475	9.9	470	9.3
493	15.5	488-489	12.3
511	8.9	508	8.3
527	13.7	521	9.9

The extinctions in Table VI are uncertain to the extent of $\pm 10\%$ at the maxima in the visible region.

Unfortunately, our molecular extinction coefficients at the maxima and minima (Fig. 3) cannot be directly compared with the data given by Karrer and Würzler. In their curve (Fig. 5 in (5)) the ordinate is designated as "log ϵ " probably because of a misprint. A calculation of the molecular coefficients in hexane based on those ordinate values would, however, yield values which are much too low. Furthermore, a high *cis*-peak at 386 $m\mu$ in the curve mentioned points to the presence of large amounts of isomerized pigment. Of course, the unusually high rates of spontaneous isomerization of the main purple bacterium pigment and the spectroscopic implications of this phenomenon were then unknown.

In order to establish the height of the *cis*-peak of neospirilloxanthin A in hexane, a chromatogram was washed with hexane, the neo A zone was eluted with abs. alcohol and the pigment was transferred into hexane, the concentration of which was established after iodine catalysis. The average value of three independent experiments at 384 $m\mu$ was, $E_{1\text{ cm.}}^{\text{mol.}} = 5.1 \times 10^4 (\pm 3\%)$; cf. Fig. 6.

The curve given for the iodine equilibrium mixture in the *cis*-peak region is the average of four independent experiments. Their greatest variation from the average at the maxima was $\pm 3\%$.

(c) *Carbon disulfide solutions.* (Figs. 4, 5; Table VII.) Since the dissolution of spirilloxanthin crystals requires some time, rapidly prepared and filtered solutions were used for the all-*trans* curve (Fig. 4). The concentrations were calculated on the basis of the extinction values at 528 $m\mu$, after iodine catalysis. In order to obtain reference values for such readings, two weighed samples were dissolved in warm carbon disulfide; after the addition of iodine and illumination the molecular extinction coefficient at 528 $m\mu$ was, $E_{1\text{ cm.}}^{\text{mol.}} = 10.3 \times 10^4 (\pm 1\%)$.

The all-*trans* curve and the iodine equilibrium curve (Fig. 4) above 450 $m\mu$ are the average of three and two independent experiments respectively with maximum

TABLE VII

Molecular Extinction Coefficients of Spirilloxanthin at the Maxima (italicized) and Minima in Carbon Disulfide

Fresh solution		After iodine catalysis	
m μ	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$	m μ	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$
390	1.32	375	1.87
		400 ^a	2.28
418 ^a	1.73	416	2.59
		430	2.34
500	8.5	500 ^a	7.8
508	8.4		
532	11.8	528	10.3
553	8.0	551	7.6
570	9.8	562	8.1

^a Point of inflection.

deviations of $\pm 2\%$. In the *cis*-peak region the all-*trans* curve with the lowest extinctions was chosen. The corresponding iodine curve was selected arbitrarily from six experiments.

The extinction data obtained in different carbon disulfide-benzene mixtures (Fig. 5) are presented in Table VIII.

TABLE VIII

Alteration of the Molecular Extinction Maxima of Spirilloxanthin in Benzene Upon the Addition of Carbon Disulfide

Per cent carbon disulfide in the solution	Cis-peak		Highest maximum	
	m μ	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$	m μ	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$
0	395	3.5	505	12.4
5	397	3.3	505	12.2
20	400	3.0	510	12.0
50	406-407	2.9	516	11.8
100	416	2.6	528	10.3

IV. Stereoisomerization of Spirilloxanthin

(a) *Cis-trans isomerization on standing or refluxing.* When a dilute solution of spirilloxanthin in benzene was kept in darkness at 23°, and then chromatographed, the ratio, unchanged all-*trans* pigment : stereoisomers, as determined colorimetrically, changed in the following manner:

Time:	3 min.	30 min.	2½ hours	5½ hours	24 hours	48 hours
Ratio:	95 : 5	92 : 8	86 : 14	83 : 17	77 : 23	75 : 25

The rate of spontaneous stereoisomerization of neospirilloxanthin A was found to be still higher; *e.g.* within 9 minutes at 10° the following ratios, all-*trans* : unchanged neo, were obtained in two experiments: 53 : 47, and 56 : 44.

A solution of 2.5 mg. of all-*trans* pigment after 30 min. refluxing yielded the ratio, all-*trans* : neo-forms = 64 : 36, and 59 : 41, in two experiments. The amount of neo B was approximately 6% of the equilibrium mixture. The chromatogram (column, 20 × 3.8 cm.) in the first experiment had the following appearance (the figures on the left denote the width of the zones, in mm.):

4 two thin pink streaks
 20 almost colorless
 30 dark rose-red, unchanged all-*trans*: 546.5, 508.5, 478 mμ
 40 reddish orange, neo A: 542, 503.5, 472 mμ
 25 orange, neo B: 537.5, 497, 462 mμ

The progress of the spontaneous isomerization of spirilloxanthin in benzene is illustrated in Table IX by the gradual increase of the *cis*-

TABLE IX

Gradual Increase of the Cis-peak of Spirilloxanthin in Benzene Solution Caused by Spontaneous Isomerization at Room Temperature

Time after dissolution	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$ at the <i>cis</i> -peak	$E_{1\text{ cm.}}^{\text{mol.}} \times 10^{-4}$ at 510 mμ
2½ min.	1.62	14.8
15 min.	1.84	14.6
30 min.	2.01	14.4
1 hour	2.15	14.3
2½ hours	2.96	14.4
4 hours	3.27	14.2
21 hours	3.27	12.5

peak. For each of these measurements fresh samples of a solution were taken while the main volume was kept in darkness. The position of the peak shifted from 397 mμ to 394 mμ in the course of a day. The estimation of the concentration of such solutions was based on the extinction value measured at the highest maximum of the all-*trans* solution. Within reasonable limits the values listed in Table IX agree with the chromatographically established extent of isomerization. The high rate of spontaneous isomerization of all-*trans*-spirilloxanthin in benzene was not diminished by the addition of the following substances (*cf.* Strain (12)): aniline, dimethylaniline, diphenylamine, *m*-phenylenediamine, 2-aminopyridine, benzidine, quinine, catechol,

glacial acetic acid, sulfur, selenium. The substitution of quartz for glass had no stabilizing effect.²

(b) *Cis-trans isomerization by iodine catalysis at room temperature.* To a solution of 3 mg. of spirilloxanthin in 100 ml. of benzene 0.03 mg. of iodine (in benzene) was added after which the solution was either kept in diffuse daylight at 22–24° for thirty minutes or illuminated for fifteen minutes as described above. There was no essential difference in the results obtained by these two methods. The chromatogram (20 × 3.8 cm.) was as follows:

- 4 two colored streaks, irreversible
- 20 almost colorless
- 40 dark rose-red, unchanged all-*trans*: 545, 507, 477 m μ
- 75 light reddish orange, neo A (+B): 538.5, 500.5, 469 m μ

In two independent experiments the colorimetric ratio was, unchanged all-*trans* : neo A(+B) = 57 : 43, and 54 : 46. The corresponding weight ratio as established by spectrophotometric readings after iodine catalysis was 60 : 40.

(c) *Cis-trans isomerization by melting crystals.* Spirilloxanthin when kept a little above its melting point (at 225°) bleaches out within a minute. Therefore, the experiments on isomerization in the melted state were carried out with mixtures of 1 part of pigment with 4 to 5 parts of naphthalene. Each sample (3 to 4 mg.) was sealed under carbon dioxide in a narrow pyrex tube, kept in a dibutyl phthalate bath at 150° for one minute and then cooled rapidly in ice water. The material was dissolved in cold benzene and developed immediately on a column (20 × 3.8 cm.):

- 1 pink, irreversible
- 15 almost colorless
- 30 dark rose-red, unchanged all-*trans*: 546.5, 508, 478 m μ
- 45 reddish orange, neo A: 540, 502, 470 m μ
- 3 almost colorless
- 20 orange, neo B: 531.5, 494, 461 m μ

² In connection with the standing experiments described, the following unexplained observation was made. When spirilloxanthin in benzene was kept at room temperature in a carbon dioxide atmosphere for four hours, a subsequent chromatogram showed, in addition to the usual zones, a minor pigment which was adsorbed above all-*trans*-spirilloxanthin, and which could be eluted with alcohol-benzene or acetone-benzene only with difficulty and partially. The formation of such a zone did not take place if the solution was kept in air or under nitrogen. The pigment mentioned showed visual maxima in benzene at 547.5, 508.5, 479 m μ ; with iodine no marked change occurred.

When the heating was continued for thirty minutes a very different picture appeared:

- 10 almost colorless
- 16 dark rose-red, unchanged all-*trans*: 545.5, 508, 477.5 m μ
- 2 almost colorless
- 16 reddish orange, neo A: 539.5, 501.5, 468 m μ
- 2 almost colorless
- 5 orange, neo B: 531, 494, 461.5 m μ
- 20 almost colorless
- 23 yellow, irreversible: 494.5, 462.5, 434 m μ
- 17 almost colorless
- 25 light yellow, neo-form of pigment in 23 mm. zone: 488, 457, 426 m μ

The colorimetric ratio was, unchanged all-*trans* : neo A : neo B = 54 : 37 : 9 after one minute melting, and 46 : 39 : 15 after thirty minutes.

In the latter case the main portion, *viz.* 60% of the recovered pigment, was composed of a new polyene, contained in the 23 mm. and 25 mm. zones, which showed maxima at wave lengths about 50 m μ shorter than those of spirilloxanthin. This yellow degradation product of spirilloxanthin was purified by re-chromatography on calcium hydroxide (developing with benzene). On the column the orange main layer (sharp visual maxima in benzene, 494.5, 462.5, 433.5 m μ) was immediately followed by a neo form (488, 457, 426 m μ), and upon iodine addition both showed bands at 492, 460 m μ . In the Beckman instrument well defined maxima were observed at 495, 463, 435-7 m μ (in benzene) which upon iodine catalysis decreased in height and shifted to 492, 460-1, 434-6 m μ . Simultaneously a moderate *cis*-peak with fine structure appeared at 350 m μ .

(d) *Photochemical cis-trans isomerization.* These experiments were carried out in transparent quartz test tubes (diam., 22 mm.) filled with carbon dioxide. Because of the high rates of spontaneous isomerization in benzene in darkness, the effect of sunlight was not as spectacular as in similar experiments with sterically more stable carotenoids (20). For example, when a solution was insolated for 30 min. the extent of isomerization was only 8 to 11% greater than when the solution was permitted to stand in darkness. The chromatograms were like those obtained by refluxing. In this case colorimetric measurements did not show any considerable loss. After one hour insolation, however, only half of the initial colorimetric value was recovered. No marked amounts of irreversibly formed pigment appeared in the chromatograms.

SUMMARY

Spirilloxanthin, $C_{40}H_{54}(OCH_3)_2$, the main carotenoid pigment of the non-sulfur purple bacterium, *Rhodospirillum* (*Spirillum*) *rubrum*, and probably identical with Karrer and Solmssen's rhodoviolascin, has been submitted to a spectroscopic and stereoisomeric study. Its molecular extinction curves are given in benzene, hexane, and carbon disulfide for the natural and isomerized pigment. Spirilloxanthin is converted by iodine (in light), or by thermal or photochemical methods into a mixture of *cis-trans* isomers in which neospirilloxanthin A predominates; the latter is adsorbed immediately below the natural pigment on the Tswett column. The rate of the spontaneous isomerization of spirilloxanthin at room temperature is higher than that of any carotenoid investigated so far. Isomerization of spirilloxanthin in benzene or hexane produces a characteristic *cis*-peak (with fine structure) in the ultraviolet region. In carbon disulfide solution, however, no steep *cis*-peak is noticed, only an elevation of the curve. Natural spirilloxanthin is an all-*trans* compound. The chromophore of neospirilloxanthin A probably contains two *cis* double bonds, one of which is centrally located.

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Purified Prothrombin: Factors which Influence Its Activation

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INTRODUCTION

The conversion of prothrombin to thrombin has long presented some of the most important unsolved problems in the blood clotting mechanism. With the use of purified clotting factors we have found it possible to gain information concerning certain of the unknown variables. It is the purpose of this paper to discuss the influence of calcium, strontium, magnesium, barium, and other salts, as well as the effect of pH, on the conversion of purified prothrombin to thrombin.

General Methods

Prothrombin. Purified prothrombin was prepared according to methods previously developed and described (1). The protein was dissolved in oxalated saline (0.9% NaCl + 0.075% $K_2C_2O_4$) and buffered with imidazole (2). Only solutions with an activity of 8,000 or more Iowa units per ml. were used.

Thromboplastin. Purified material was made from beef lung extract (2). One ml. of such preparations have been found, consistently to have the ability to transform at least 200,000 units of prothrombin to thrombin. On the basis of previous work (7) a unit of thromboplastin can be defined as that amount which will convert one unit of prothrombin to thrombin, when the prothrombin is in excess, and all other factors which influence the reaction are controlled in a manner which will permit the reaction to go to completion. We have characterized the preparation with respect to activity. It contained 200,000 units per ml. On a stoichiometric basis this clotting factor was allowed to react with prothrombin in the ratio of 40:1 units respectively.

Purified Prothrombin Assay. 0.3 ml. of prothrombin was mixed with 0.1 ml. thromboplastin and 0.1 ml. 0.6% calcium chloride in physiological saline solution. After 10 minutes the thrombin concentration was measured quantitatively with the use of standardized fibrinogen solution (3).

Prothrombin, Thromboplastin Reaction. It is possible to stop this reaction at any time by adding a neutral oxalate ion. In practice it is convenient to dilute a small

amount (0.1 ml.) of the concentrated reaction mixture with oxalated saline solution, the dilution being governed by the original concentration of the reaction mixture. This blocks the reaction, and at the same time the diluted solution can be assayed for thrombin directly. Only one precaution is necessary. Standard procedure requires that thrombin be assayed in the presence of calcium ion (3). Consequently all results were uniformly low. It was, therefore, necessary to multiply by the correction factor of 1.5 to obtain standard units (3).

When a new environment is imposed on the reaction between prothrombin and thromboplastin two results are of prime interest, (a) the reaction rate, and (b) the final equilibrium conditions. Preliminary experiments showed that the thrombin concentration at five minutes is a sensitive index of the reaction rate, and that the reaction comes to equilibrium in less than three hours. Accordingly the practice of measuring the thrombin concentration at five minutes and at three hours was followed throughout.

Salts. High grade products were used without further purification.

EXPERIMENTAL

Effect of pH. Prothrombin solutions were made acid with 0.1 *N* HCl, or alkaline with 0.1 *N* NaOH, and the pH measured with the glass electrode. Volume corrections for acid or alkali additions were made. Thromboplastin solutions of corresponding pH were also prepared. Prothrombin and thromboplastin solutions of identical pH were mixed, and CaCl₂ was added to give the optimum concentration of the latter.

It was found (Chart 1) that the optimum pH is approximately 7.2. After final equilibrium there was complete transformation of prothrombin to thrombin within the limits of pH 6.2 and 8.7. Beyond these limits the yield of thrombin was markedly decreased and there was virtually none at either pH 5 or 10. These latter points are very near the pH values found for the inactivation of prothrombin with acid or alkali (1).

Calcium Requirements. These experiments were performed at pH 7.2. 0.5 ml. prothrombin and 0.1 ml. thromboplastin were mixed. The K₂C₂O₄ in the prothrombin preparation was taken into account; and, in serial experiments various amounts of CaCl₂ (in 0.4 ml. volume) were added to give various concentrations of calcium in the final mixture of 1 ml. It was found that just sufficient calcium to neutralize the K₂C₂O₄ (Chart 2) already permitted the reaction to go rapidly. The rate at which thrombin was produced and the final quantity of thrombin produced compare favorably with optimum calcium concentration, which was found to range from near zero to 0.0075 *M*. It was also found that when the calcium concentration was greater than

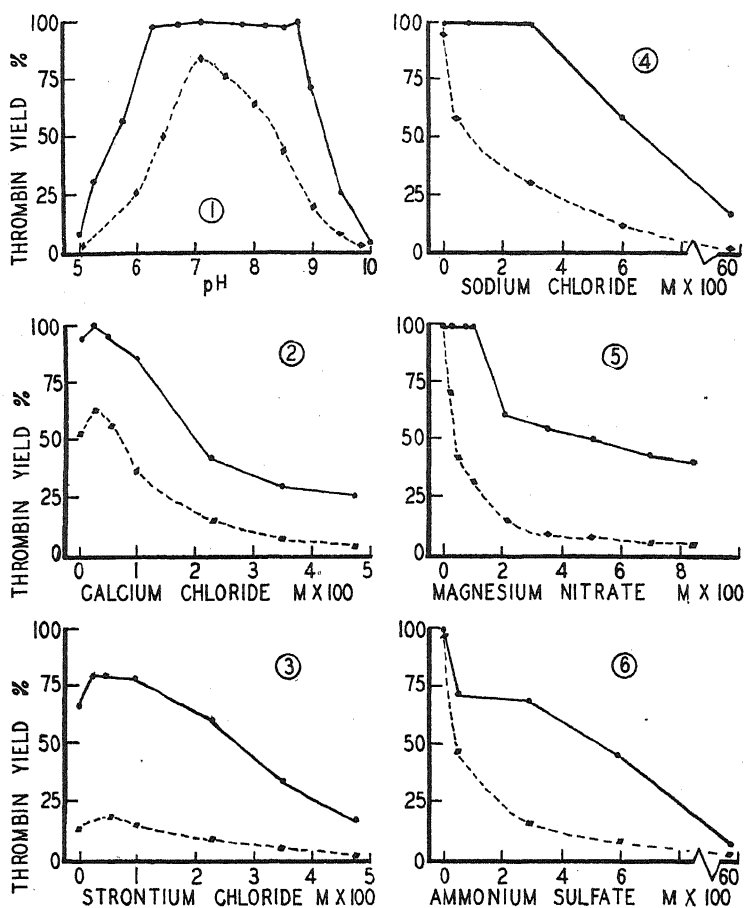


FIG. 1

Factors Which Influence the Activation of Purified Prothrombin at 28°C.

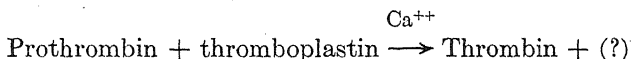
The solid lines represent equilibrium conditions as found at the end of 3 hours. The thrombin concentration at the end of 5 minutes is represented by broken lines, and reflects primarily the rate of the reaction.

1. Influence of pH changes.
2. Influence of calcium concentration.
3. Substitution of strontium for calcium.
4. Inhibitory effect of NaCl with optimum calcium and pH.
5. Inhibition with magnesium, in the presence of optimum calcium and pH.
6. Influence of $(\text{NH}_4)_2\text{SO}_4$ at pH 7.2.

on that idea. We have yet to encounter spontaneous conversion of any appreciable quantity of prothrombin.

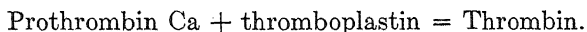
DISCUSSION

A review of these experiments makes it difficult to escape the conclusion that the function of calcium is to act as a catalyst. It is primarily concerned with the rate of the reaction. Strontium can serve the same purpose, but so poorly that a side reaction (2) has sufficient time to destroy some prothrombin. If it can ultimately be shown that calcium or strontium do not become an integral part (not simply a salt) of the thrombin molecule or an integral part of any other product of the reaction the evidence will be complete. Unfortunately the work required to establish such information will be quite considerable. Meanwhile it seems judicious to write the equation as follows:

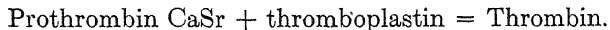


This represents the calcium ion as a catalyst and the reaction to be stoichiometric in character (7).

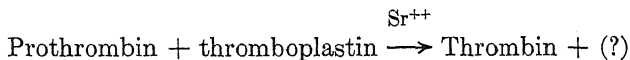
The equation introduced by Quick (8) has the following form:



This embodies his conclusion "that prothrombin itself is a calcium compound." If his view is correct how is one to explain the activation of our material with strontium? We know that the highly purified material which we isolate and activate is prothrombin, a protein, because it will yield thrombin. It is not logical that, by adhering to Quick's suggestion, we represent the activation with strontium as follows:



As with calcium we prefer to write



Furthermore it seems to us that the minimum requirements to prove that prothrombin is itself a calcium compound calls for knowing its molecular weight and showing that there is at least one atom of calcium, not present as salt, for each molecule of protein.

We can broaden our understanding of the fact that calcium and strontium produce their catalytic effect at optimum concentrations. First, the catalyst must be present. Second, small amounts are most favorable. Third, in higher concentrations the catalyst becomes inhibitory in much the same manner as NaCl or any other neutral salt.

SUMMARY

Prothrombin reacts with thromboplastin in the presence of calcium ions most efficiently at pH 7.2. The reaction goes to completion within the pH limits of 6.2-8.7, and can be blocked at either pH 5 or 10. Strontium can be substituted for calcium, but the reaction rate is much slower than with calcium and it is difficult, if not impossible, to obtain as much thrombin when strontium is substituted for calcium. Neutral salts such as NaCl and $\text{Mg}(\text{NO}_3)_2$ inhibit the reaction. Inhibition begins at concentrations comparable to the concentrations where calcium or strontium salts are no longer at optimum concentration, and begin to be inhibitors of their own catalytic function. It is pointed out that calcium serves the purpose of a catalyst.

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A Dietary Factor, Essential for Guinea Pigs

IV. Serum Phosphatase During Deficiency¹

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It was shown previously (1) that guinea pigs developed a deficiency disease, characterized by degenerative changes in skeletal musculature when the animals were raised on a diet composed of skimmed milk to which had been added 10% of skimmed milk powder, adequate amounts of copper, iron, carotene, and orange juice. The first sign of the deficiency was the development of stiffness at the wrist joint. In later stages of the deficiency deposits of calcium phosphate were found in almost every body tissue. A highly active fraction was isolated from raw cream which in a daily dosage of 0.1 γ was able to cure the stiffness induced by the skimmed milk diet within five days.

A deficiency of the anti-stiffness factor also resulted in an abnormal distribution of the acid soluble phosphorus in the liver and kidneys of the experimental animals (2). The most striking changes observed were the increase of the inorganic phosphorus and the decrease of the easily hydrolyzable phosphorus during the deficiency. It was thought that the high concentration of inorganic phosphorus could be the cause for the excessive calcium deposits which appeared in almost every body tissue. In this connection it was of interest to determine the alkaline phosphatase activity of the blood serum of guinea pigs during the different stages of the deficiency.

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²Based in part on data from a thesis submitted by one of the authors (A. S. R.) as partial fulfillment of the requirements for the degree of Master of Science, Oregon State College.

Changes in the alkaline phosphatase content of serum and plasma have been reported during vitamin D deficiency, in bone diseases and during vitamin C deficiency. It is a well-established fact that the phosphatase content of the blood serum is invariably increased far above normal in the case of rickets. One of the first signs of a vitamin D deficiency is the increase of serum phosphatase (3, 4). The high level does not decrease immediately after the administration of vitamin D and may not reach a normal level for several months after healing from vitamin D deficiency becomes evident (5, 6).

In bone diseases, such as osteomyelitis, osteitis deformans and arthritis where bone changes are involved the average phosphatase content of the serum is reported to be slightly higher than that of normal individuals (7, 8).

Shwachman and Gould have reported a decrease in the serum phosphatase during experimental scurvy. After administration of adequate amounts of *l*-ascorbic acid a return to the normal phosphatase level was observed (9, 10, 11).

It was found in this investigation that a deficiency of the anti-stiffness factor also resulted in a lowering of the phosphatase level.

EXPERIMENTAL

Two different sets of animals and two methods of analysis were used during this investigation.

In the first series of experiments the animals were raised on the diet as described by van Wagtendonk and Wulzen (1). The diet has the following composition:

Skimmed milk	100	cc.
Skim milk powder	10	g.
Ferric chloride	0.82	mg.
Copper sulfate	0.78	mg.
Orange juice	1	cc./100 g. body weight/day
β -carotene	150	I.U./day
Straw		<i>ad libitum</i>
Iodized salt		<i>ad libitum</i>

The method of King and Armstrong (13) was used for the determination of the phosphatase activity. Guinea pigs of different age groups raised on a "stock" diet (rolled barley, grains *ad libitum*) were compared with corresponding animals which had received the deficient diet. Groups of 10 animals were used for analysis. The blood was collected by cardiac puncture under slight ether anesthesia. It was sampled in oxalated tubes and centrifuged for 10 minutes at 4000 r.p.m. The results are given in Fig. 1. The serum phosphatase values of the deficient guinea pigs showed

the same trend as those on the stock diet, but on a lower level throughout the experiment.

In the second series of experiments (C.E.B.) the animals were raised on the diet described by van Wagtenonk (2). This diet is composed as follows:

Skim milk powder	16 g.
Water	84 g.
Ferric chloride	0.25 mg.
Copper sulfate	0.25 mg.
Autoclaved straw	<i>ad libitum</i>
Iodized salt	<i>ad libitum</i>

The diet was given twice a day. To the morning feeding a solution of the water-soluble vitamins was added in such a concentration that the average daily intake

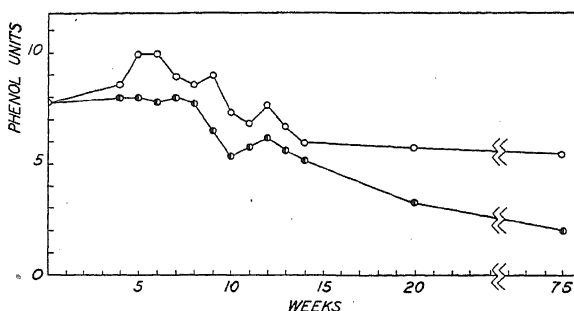


FIG. 1

The Alkaline Serum Phosphatase of Guinea Pigs Raised on a "Stock" Diet and on a Diet Deficient in the "Anti-Stiffness" Factor

○ = stock diet; ● = deficient diet

per animal of the individual vitamins was: Thiamin hydrochloride 0.2 mg.; pyridoxin hydrochloride 0.1 mg.; riboflavin 0.5 mg.; nicotinic acid 1 mg.; pantothenic acid 0.1 mg.; inositol 10 mg.; *p*-aminobenzoic acid 2 mg.; choline 50 mg.; biotin (S.M.A. concentrate S 200) 0.01 mg. To the evening diet was added a solution of the fat-soluble vitamins in cottonseed oil. The average daily intake per animal was: β -carotene 150 I.U.; viosterol 40 I.U.; α -tocopherol 0.1 mg.; 2-methyl-1,4-naphthoquinone 0.1 mg. Ascorbic acid (50 mg.) was administered orally once per week.

The phosphatase activity was determined by the method of Shinowara, *et al.* (14) as modified by Gould and Shwachman (12). The animals (groups of 15 guinea pigs were used) were anesthetized (nembutal) and the blood removed by cardiac puncture. Heparin was used to prevent clotting. The influence of ageing on the serum phosphatase level both in normal and in deficient animals had been demonstrated in the first experiment. It was therefore considered unnecessary to make a rigorous

comparison between the normal and deficient animals of different age groups. The results of this experiment are summarized in Table I. The same picture was ob-

TABLE I

Alkaline Serum Phosphatase of Guinea Pigs Raised on a "Stock" Diet and on a Diet Deficient in the Anti-Stiffness Factor

Age in weeks	Diet	Weeks on diet	No. of determ.	Mg. P. liberated per 100 cc. serum <i>Mean</i>
13	stock	13	15	10.7
20	stock	20	13	8.8
65	stock	65	10	2.8
14	skimmed milk	1	15	7.5
16	skimmed milk	2	15	6.5
20	skimmed milk	7	15	5.3
41	skimmed milk	28	15	2.8
65	skimmed milk	52	15	0.4
24	skimmed milk ^a *	11	15	7.6
20	skimmed milk ^b *	7	15	5.3

^a 1000 Units of the anti-stiffness factor every other day during the whole course of the experiment.

^b 1000 Units of the anti-stiffness factor during the last five days of the experiment.

* One unit was arbitrarily defined as follows: A solution of the compound in Wesson oil contains one unit per cc. if, when 1 cc. is administered daily for five consecutive days to a deficient animal, it alleviates the induced stiffness in this time.

tained as in the first experiment. Shwachman and Gould reported a drop in the serum phosphatase level during scurvy and a return to normal values after administration of ascorbic acid. As can be seen from Table I the serum phosphatase in deficient animals did not return to a normal value after administration of the anti-stiffness factor.

DISCUSSION

It is evident from the results reported in this paper and in the previous publication (2) that a deficiency of the anti-stiffness factor causes a serious derangement of the phosphorus metabolism. The normal distribution of the acid-soluble P in the liver and the kidneys is disturbed during the deficiency while the alkaline serum phosphatase decreases. The only other deficiency disease for which a decrease in the serum phosphatase has been reported is scurvy. It is interesting to note that the scorbutic diet as employed by Gould and Shwachman contains 50 per cent heated skimmed milk powder. It might be suggested that both the diets used in the present investigation were not

adequately supplied with ascorbic acid. However, the animals in the second experiment received 50 mg. of *L*-ascorbic acid once per week. According to Zilva (15) 35 mg. every seven days furnishes adequate protection against scurvy. The amount used in this experiment is well above the minimum preventive dosage. The animals did not show any sign of scurvy. This was also the case in the first experiments, where the animals received ample amounts of orange juice. The decrease in the serum phosphatase level can therefore be attributed to an absence of the anti-stiffness factor from the diet. Shwachman reported that in his experiments the low level of serum phosphatase returned to normal in five days after administration of 0.225 mg. of *L*-ascorbic acid per day. In the experiment reported here administration of the anti-stiffness factor had no immediate influence on the low level of the serum phosphatase, not even in dosages which are as much as 1000 times the dosage required to alleviate the stiffness and to normalize the abnormal distribution of the acid-soluble P in the liver and kidneys of the guinea pigs deficient in the anti-stiffness factor. It is questionable whether a still larger dosage would be effective. It is more likely that the behavior of the serum phosphatase is analogous to that in rickets. Here it is reported that the serum phosphatase remains on a high level for a considerable length of time after the healing of vitamin D deficiency. However, a decrease of the serum phosphatase could be prevented by a continuous administration of the anti-stiffness factor.

SUMMARY

The alkaline serum phosphatase in guinea pigs, deficient in the anti-stiffness factor, is lower than in non-deficient animals of the same age group. There is no immediate return to a normal level after the administration of this factor in doses sufficient for the alleviation of other symptoms. Ascorbic acid in sufficient amounts to prevent scurvy did not prevent a drop in the serum phosphatase level. Continuous administration of the anti-stiffness factor did prevent a low level.

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Penicillic Acid from *Aspergillus Ochraceus*, *Penicillium Thomii*, and *Penicillium Suavolens*¹

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INTRODUCTION

It is rapidly becoming established that specific antibiotic substances may be produced by different species and genera of fungi. The following tabulation summarizes known examples of this type.

<i>Antibiotic agent</i>	<i>Organism</i>
Penicillin (or biologically indistinguishable substances)	<i>Penicillium notatum</i> (8)
	<i>P. citreo-roseum</i> (10)
	<i>P. chrysogenum</i> (19)
	<i>Aspergillus flavus</i> (5, 15, 21)
	<i>A. flavipes</i> (10, 25)
	<i>A. oryzae</i> (10)
	<i>A. nidulans</i> (10)
	<i>A. niger</i> (10)
	<i>A. parasiticus</i> (9)
	<i>A. giganteus</i> (17)
	<i>A. clavatus</i> (3, 12, 23, 26)
	<i>P. expansum</i> (2)
Clavacin, claviformin, or patulin	<i>P. claviforme</i> (3, 6)
	<i>P. patulum</i> (18)
	<i>P. melinii</i> (14)
	<i>Gymnoascus</i> sp. (14)
Penicillic acid	<i>P. puberulum</i> (4)
	<i>P. cyclopium</i> (4)
Citrinin	<i>P. citrinum</i> (11)
Gliotoxin	<i>Aspergillus</i> sp. (20)
	<i>Gliocladium fimbriatum</i> (24)
	<i>Trichoderma</i> sp. (24)

¹ We wish to thank Dr. C. Thom for identifying these two *Penicillia* which were isolated from soil.

Fumigacin or helvolic acid

A. fumigatus (16, 22)

Penicillium sp. (13)

A. fumigatus (23)

A. fumigatus mut. *helvola* Yuill. (7, 22)

EXPERIMENTAL

This paper supplements the list by showing that penicillic acid is formed by *Aspergillus ochraceus*² and *Penicillium thomii* and *P. suavolens*. Culture filtrates of the first two organisms grown for 7 days on Czapek-Dox medium containing 3% corn steep liquor inhibited *Escherichia coli* and *Staphylococcus aureus* at 1/40 and 1/80, respectively. The *Aspergillus* activity was adsorbed on norite at pH 2.0 and eluted with methanol, which, upon *in vacuo* concentration, yielded crystalline material. The substance was recrystallized twice from 50% methanol, yielding approximately 200 mg. per liter of broth.

The substance melted at 61° and is an acid with a neutralization equivalent of 191. Corresponding values for hydrated penicillic acid are 58–64° and 188, respectively. In drying *in vacuo* over P₂O₅ the material lost 9.98% water. Theoretical, 9.58%. The anhydrous substance melted at 84°. Birkinshaw, *et al.* (4) and Alsberg and Black (1) reported 87°. Neutralization equivalent of the anhydrous acid, 169. Theory, 170. The substance analyzed as follows: C, 56.70; H, 5.65. Theoretical for penicillic acid (anhydrous) C₈H₁₀O₄: C, 56.44; H, 5.93. Aqueous solutions of the crystalline product reduced permanganate in the cold, showed no color reaction with FeCl₃ and gave a purplish color with NH₄OH as described by Alsberg and Black, who first isolated penicillic acid. It did not reduce Fehling's solution. A crystalline product from the reaction of phenylhydrazine with the substance melted at 172–3° which corresponds to phenylpyrazoline hydrazone obtained similarly from penicillic acid (Alsberg and Black found 171°; Birkinshaw, *et al.* found 176°). The antibacterial action and mouse toxicity of the pure material correspond closely with those described by Alsberg and Black, and Birkinshaw, *et al.* The substance is in all respects identical with penicillic acid.

Six out of 16 strains of *Aspergillus ochraceus* tested gave culture filtrates inhibitory to *E. coli* when diluted 1/10.

The chemical and biological properties of the pure material isolated from culture filtrates of *Penicillium suavolens* by ether extraction at pH 2.0 and crystallization from the aqueous solution of the residue were identical with the *Aspergillus ochraceus* material. It melted at 59° and a mixed melting point of the two showed no depression. The

² This culture was kindly supplied by Dr. Charles Thom and bore the number 260 LTL.

crystalline substance isolated similarly from *Penicillium thomii* proved to be identical with penicillic acid.

DISCUSSION AND SUMMARY

The accumulated evidence makes it likely that the formation of the known, and as yet undiscovered, antibiotic agents of mold origin is much more widespread among filamentous fungi than is generally believed, with the possibility that it may be a general phenomenon at least among certain classes of these organisms. It is felt, furthermore, that these substances are manifestations of one or more types of metabolism common to such organisms and that strain specificity in these matters may be more quantitative than qualitative in importance. It seems quite reasonable that great numbers of these organisms possess the enzyme makeup for the formation of such molecules but that the amounts formed are undetectable by the conventional methods of testing for antibacterial substances. The situation is analogous to the synthesis of vitamins by microorganisms. Some organisms synthesize and excrete certain vitamins vastly in excess of the amounts they require and in concentrations easily detectable with available methods; others synthesize extremely small amounts, comparatively. Doubtless the same holds for the synthesis of amino acids and possibly any of the numberless molecular species that microbial cells are capable of synthesizing. Recently available sensitive microbiological amino acid assays will permit a test of this idea. The chances that small amounts of antibiotics in microbial culture fluids would escape detection are considerably greater than in the case of vitamins because microbiological tests for vitamins are much more sensitive than those for most antibiotics.

The customary methods for detection of antibiotics at best can permit only a skimming of the surface in the quest for organisms producing such substances. Furthermore, usually only one or a few media are arbitrarily chosen for testing the ability of the organism to produce antibiotic substances, and the well known extreme fluctuations in amounts of these materials formed, as influenced by the composition of the medium, very likely would permit many potentially important organisms to escape notice. Obviously, a much more sensitive test for antibiotic activity in culture filtrates would make many more antibiotic substances available for examination and therapeutic evaluation.

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On the Mechanism of Enzyme Action. Part 24 Fe⁺ Formation in Fusaria

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INTRODUCTION

Previous investigations from this laboratory revealed that the genus *Fusarium* besides containing enzymes capable of alcoholic fermentation of hexoses as well as of pentoses possesses a powerful dehydrogenating enzyme system (1). The methodical deployment of this group of enzymes present in *Fusaria* lead to the elucidation of phase sequences of the dehydrogenation of primary, secondary, and tertiary alcohols (2). It was also found that *Fusaria* pigments and related compounds are able to give rise to an inhibition and acceleration of these dehydrogenations (3).

In the course of these studies it was noticed that although the mycelia of some *Fusaria* contain exceedingly small quantities of various pigments (4), the fat content (18) amounted to as much as 10%³ of the dry mat weight. In view of the discovery of a connection between pigment structure and the rate of dehydrogenation of isopropyl alcohol to acetone, it seemed to be requisite to attempt the elucidation of the chemical composition of this fat as a basis for a warrantable investigation of a relation between a possible mechanism of its formation in the cell through the action of enzymes so far not accounted for in *Fusaria*.

¹ Condensed from a part of the dissertation of R.P.M. submitted to the Graduate Faculty of Fordham University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The results of this investigation were presented before the Division of Biochemistry at the autumn meeting of the American Chemical Society, New York, N. Y., 1944.

² Communication No. 34.

³ See: *Arch. Biochem.* 4, 423 (1944), Table I.

EXPERIMENTAL

Cultures employed. The following *Fusaria* were used in these investigations: *Fusarium graminearum* Schwabe (Fgra) No. A36-1-VIII, obtained from the University of Minnesota through the courtesy of Dr. C. J. Eide; *Fusarium lini* Bolley (FIB) No. 5140, obtained from the Biologische Reichsanstalt, Berlin-Dahlem, through the courtesy of Dr. H. Wollenweber. Stock cultures of these fungi were maintained on the usual nutrient medium (5).

All cultures were transferred at two-week intervals and periodically examined to check their purity.

Procedures. All the enzymatic experiments were conducted by growing *Fusaria* in 125 ml. Erlenmeyer flasks, each flask containing 50 ml. of nutrient medium. These media, consisting of the usual inorganic salts together with either a fatty acid or olive oil, were emulsified by the use of a household homogenizer with 0.05% polyvinyl alcohol (high viscosity-type B: Du Pont) (6) as the emulsifying agent. Polyvinyl alcohol possessing long-chain molecules was found to be far superior to other stabilizing agents, *e.g.* gum arabic, carob bean gum (7) etc. The initial pH in all cases was 4.4. However, in evaluating analytically results obtained with emulsions prepared by a simple household homogenizer instead of a Hurrell laboratory homogenizer it has to be borne in mind that two basic requirements of stable emulsions could be not met: There was (a) a lack of satisfactory regulation of the conditions of agitation and (b) the unavoidable period and temperature (8) of incubation of the culture media. Since emulsions usually contain dispersed globules of greatly varying diameter, the desired reduction of their size to an approximately equal diameter is dependent on the efficiency of homogenization. Consequently, the exposure and surface of the available and enzymatically attacked substrate is within limited control.

The procedure used when preparing a uniform spore-mycelial suspension of the microorganism for the inoculation of stock cultures and experiments was the same as that employed previously in related investigations (9, 10). The media were sterilized at 15 pounds pressure for 15 minutes at 120° C. and all cultures were grown in the dark under aerobic conditions at 28°.

Mycelial weights were determined, when required, by filtering the mycelia from the nutrient media, washing with three successive 10 ml. portions of cold petroleum-ether, followed by 15 ml. of distilled water, and drying over night in an oven at 60°.

The constants for the fats were determined according to standard methods (11) after separating the nutrient media from the mycelia, acidifying with HCl, extracting with petroleum-ether; drying over anhydrous Na₂SO₄, and evaporating to dryness

in a current of CO₂. All analyses were run in duplicate using an aliquot of the substrate obtained from ten flasks. The mycelial weights in the enzymatic experiments represent an average of the mat weights obtained from five flasks (containing 50 ml. of nutrient medium per flask) after fourteen days of growth.

Isolation and Analysis of Fusaria Fats. The crude mixture of fats was obtained by growing *Fgra* on a Raulin-Thom nutrient medium in a steriliincubator for three weeks, removing the mycelia, washing with H₂O and drying in air with the aid of an electric fan. These mats were then treated in a Soxhlet extractor using petroleum-ether. The material so obtained was subjected to further investigation.

Wax Fraction. The petroleum-ether was removed in vacuo; the residue dissolved in ether and diluted with an equal volume of acetone. The failure of a precipitate to form indicated the absence of phosphatides in the mixture. The ether was thereupon evaporated in vacuo, and the solution cooled in ice. The precipitate which formed, amounting to approximately 13% of the original weight of crude fat, consisted of a brown-red solid possessing an aromatic odor.

Acetone-Soluble Fraction. After separation of the above precipitate by filtration, the solution was concentrated to dryness in vacuo, and the residue, amounting to 81% of the original fat, consisted of a dark brown semiliquid mass having an iodine value of 84.63 and a saponification number of 189.23. These constants compare favorably with those for oleic acid and olive oil (12).

Saponification of the Fat. Twenty-five grams of fat was saponified by refluxing for five hours with 300 ml. of 4% alcoholic potassium hydroxide. After concentrating to half its volume, the solution was diluted with 500 ml. of water and extracted five times with ether. The ethereal extract was concentrated to dryness, and the residue again refluxed for one hour with alcoholic potassium hydroxide. The solution was diluted with water and extracted with three portions of ether, and the ethereal solution again washed with water. The aqueous solution and the washings were combined with the original soap solution.

The unsaponifiable matter was obtained by concentrating the ethereal solution and drying the residue. It formed a thick yellow oil amounting to 2.1% of the acetone-soluble fat.

The aqueous soap solution was acidified with hydrochloric acid, and the fatty acids were extracted with three portions of ether. After being washed with water until free from hydrochloric acid, the ether solution was dried over anhydrous sodium sulfate, filtered, and distilled. The residue solidified to a semiliquid yellow-orange mass amounting to 96.3% of the acetone-soluble fat and having an iodine number of 84.17.

Water-Soluble Constituents. The aqueous solution, after the extraction of the fatty acids, was concentrated in vacuo to half its volume and the potassium chloride separated by filtration. The filtrate was again concentrated nearly to dryness in vacuo and the moisture removed as far as possible by evaporating the residue ten times with absolute alcohol. The residue consisted of a small amount of brown solid which was not further examined.

Separation of the Fatty Acids. The crude fatty acids, 24.1 g., were converted into lead soaps according to the procedure outlined by Twitchell (13), extracted with ether and the ether-soluble and insoluble portions were decomposed by shaking with dilute hydrochloric acid. The fatty acids after separation in this manner were found to consist of 23.3% of solid acids and 69.8% of liquid acids, the latter becoming lighter in color upon standing.

The analytical data obtained from the investigation of the *F. gra* fat are compared in Table 1 with those obtained from yeast.

TABLE I
Comparison of Fusaria and Yeast Fats

	Fusaria fat	Yeast fat (14, 15)
Iodine number	84.68	71.1
Saponification number	189.28	162
Unsaponifiable matter	2.1%	15.6%
Saponifiable matter	96.3%	ca. 84.4%

Examination of the Solid Fatty Acids. Since it was found impossible to separate the major portion of the fatty acids (1.5 g.) by crystallization, they were converted into the methyl esters (16) and subjected to fractional distillation in a vacuum of 0.01 to 0.001 mm. pressure. The main fraction amounting to about 60% of the total quantity, distilled over at 114–116°. The free acid was obtained by saponification with alcoholic KOH and recrystallized from methyl alcohol. White plates were obtained that melted at 63–64° and remained unchanged after further recrystallizations. A mixed melting point with pure palmitic acid showed no depression. The other single fractions were too small to permit further investigation.⁴

Enzymatic Action of FlB. upon Fatty Acids and Olive Oil

Effect of FlB. upon Stearic Acid. Using stearic acid as the carbon source, the dehydrogenating action of FlB. upon the fatty acid was

⁴ The composition of the liquid fatty acid portion of the mixture will be reported later.

investigated by means of determining the iodine number of the mixture of fatty acids extracted from the nutrient medium.

From an examination of the results shown in Fig. 1, it would appear that after an initial increase in the iodine values, the emulsion began to disintegrate so that the greater bulk of acid available for the organism to act upon is unsaturated. In turn the unsaturated acid is split

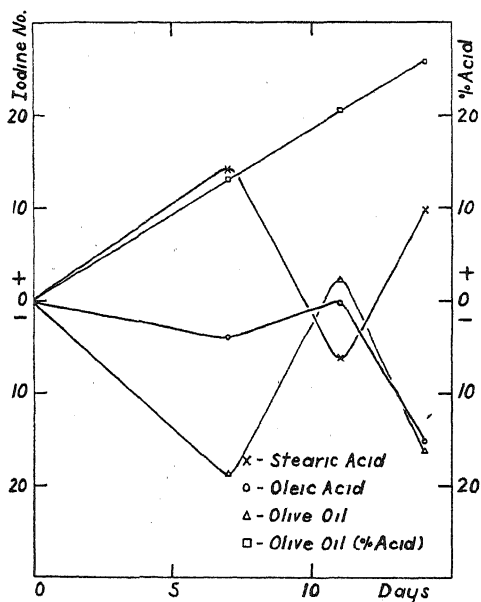
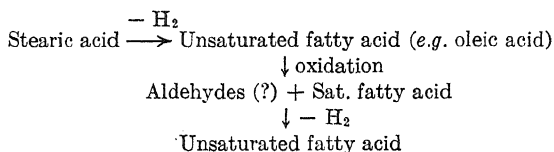


FIG. 1

by FLB. to give, among other products, a saturated acid the presence of which is indicated by a decrease in the iodine number on the 11th day. Finally the saturated acid so formed is dehydrogenated and consequently the iodine value on the 14th day again shows an increase. These reactions may be indicated by the following scheme:



Effect of FLB. upon Oleic Acid. Lang and Adickes (17), in their studies on the dehydrogenation of stearic acid by liver or muscle extracts, have reported that the oleic acid formed in the course of such an action is not further acted upon by the enzyme system employed. In distinction to these findings, when the action of FLB. upon oleic acid was studied, growth was visible within two days after inoculation.⁵ Again as in the case of the stearic acid, the breakdown of the emulsion influences the analytical interpretation of the results. The oxidation of oleic acid, apparent on the 7th day (Fig. 1) results in the formation of a saturated fatty acid and consequently causes a decrease in the iodine number. The latter acid is then dehydrogenated (increase in iodine number on 11th day) and the resulting unsaturated compound finally oxidized (decrease in iodine number on 14th day).

Effect of FLB. upon Olive Oil. As a result of the close similarity between the constants obtained from analyses of *Fusaria* fat and olive oil, the action of the organism upon an emulsion of the latter was investigated. As in the case with oleic acid the results depicted in Fig. 1 are similar to those obtained with the acid but more pronounced. An interpretation of these results on the basis of iodine values alone is complicated because of the presence of the various saturated and unsaturated fatty acids as glycerides in the original olive oil. The increase of free fatty acids when using olive oil, however, indicates the existence of lipase activity in FLB.

DISCUSSION

The problem of the synthesis of lipids from hexoses and especially from pentoses is rather complex and intriguing. The large quantity of fat (18) produced in the course of growth of *Fusaria* on various media (18a) seems to have a definite function. No doubt these fats are synthesized by means of a lipase system and, from the information thus far available, the fat consists mainly of an unsaturated fatty acid similar to oleic acid. The study of an enzymatic action of FLB. upon a likely precursor, such as stearic acid, indicates that the saturated acid may be dehydrogenated by an enzyme system similar to that by which it was formed in *Fg*, perhaps giving rise to aldehydes (generally expressed) in accordance with the findings of Feulgen and Bersin (19), and to a

⁵ The average mat weights obtained in cultures containing oleic acid and olive oil as carbon source were 102.8 and 33.9 mg. respectively.

saturated acid. The saturated fatty acid thus obtained can be further acted upon by the enzyme system present. Thus, presumably, the precursor of the end product formed, perhaps, in agreement with the postulation of Witzemann (20), is not a static entity but in reality undergoes a series of changes thus playing an integral role in the total metabolism of the organism.

From a comparison of the analytical constants it would appear that the composition of yeast fat (14) and of *Fusaria* fat are similar. Moreover *Fusaria* possess also a fatty acid dehydrogenase, and are able to split olive oil, just as it was observed with yeasts (21). On the other hand the earlier established ability of *Fusaria* to utilize elementary sulfur as a hydrogen acceptor (22) in distinction to certain chemo-autotrophic bacteria and their failure to give rise to the formation of phosphoglyceric acid while fermenting pentoses via pyruvic acid, seem to clearly demonstrate the great variety of general pathways which nature is capable of mustering in microbiological syntheses and degradations.

Acknowledgment: This investigation has been supported by a grant of the Rockefeller Foundation. The authors are indebted also to the Rohm and Haas Company for assistance rendered.

SUMMARY

1. *Fusarium* fat contains palmitic acid and a large portion of unsaturated liquid acids resembling those present in olive oil.
2. FIB. possesses fatty acid dehydrogenases capable of acting on both saturated and unsaturated fatty acids.
3. *Fusaria* contain a fungus lipase.
4. The bearing of the above facts on fat formation in growing *Fusaria* is discussed.

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Relation of Diaphorase to Cell Growth

Prior to discussing the clinical value of various respiratory enzymes in the application to wound healing, it was deemed of primary importance to study their effects upon growing cells in tissue cultures. This report is restricted to the effects elicited by flavoprotein enzyme (diaphorase) alone, and in combination with coenzyme 1 and amino-acid metabolites upon chick, adult mouse, and adult human fibroblasts.

We are indebted to Dr. David E. Green for preparing the pure enzyme possessing the prosthetic group, flavinadenine dinucleotide from pig heart according to the method of Straub (1).

In order to determine the stimulating effects of diaphorase alone and in combination with coenzyme 1 and amino-acid metabolites upon cell proliferation, a comparison was made with an adequate number of untreated tissue controls. A total of 187 slide and 37 Carrel flask experiments were performed to estimate the degree of stimulation. Of these total experiments, 50 slide and 14 Carrel flask experiments served as controls.

A concentration, 7.8 $\mu\text{g.}$, of this flavoprotein enzyme (diaphorase) per ml. of fluid menstruum (substrate) was found to be toxic to all the cells in the cultures. On the other hand, one tenth the concentration (0.78 $\mu\text{g.}$ of this enzyme per ml. of substrate) exerted a stimulating effect on the cells. This proved to be the concentration for maximal proliferation while 0.39 $\mu\text{g.}$ of diaphorase per ml. of substrate gave the minimal effective response. The addition of coenzyme 1 enhanced the effects produced by diaphorase alone. Various amino acid metabolites (glutamic acid, alanine, and creatine) were added to this enzyme system and their effects upon cell proliferation noted. Cell growth was found to be responsive to creatine. Creatine in minimal concentration gave a most pronounced synergistic effect when applied with the minimal dose of diaphorase found to be effective alone. When the maximal effective dose of diaphorase was employed, the additive effects of creatine were slight. We observed that diaphorase stimulates also the migration of wandering cells.

The prosthetic group, flavinadenine dinucleotide derived from yeast was without effect on the fibroblasts. This reaffirms the significance of the protein moiety in determining the activity of the diaphorase.

The substrate fluid containing diaphorase and coenzyme 1, after bathing the fibroblasts, was collected and micro-analyses made. The fluid was found to contain appreciable amounts of H_2O_2 and lactic acid. The lactic acid was determined by a recent method (2). An attempt is now being made to determine whether pyruvic acid is present in the substrate fluid.

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LETTER TO THE EDITORS

Production of a Creatine-Creatinine Destroying Enzyme from a Urine Mold

During a recent study of creatine and creatinine excretion in the rat the diluted urine samples were allowed to stand in their beakers for several days at room temperature (1). At this time a grayish-white mold was observed growing on top of the urine. It was reasonable to believe that creatinine, among other urine compounds, might furnish the nitrogen necessary for the growth of the mold. This point was studied, and an enzyme was produced which destroyed creatine and creatinine under aerobic conditions. The mold was separated from the urine and washed out several times in the centrifuge with distilled water. It was then worked up by the technique described in our last paper on the subject (2). Different transfers of the material were labelled UE_1 to UE_5 (UE = urine enzyme). The creatinine in mg. per hour destroyed in transfers 2 through 5 was as follows: 0.53, 0.52, 0.48, 0.26, 0.33. The average was 0.42 mg. per hour.

It was noticed that the activity was gradually lost in the fourth and fifth transfers, and UE_5 did not destroy any more creatinine. Something else besides creatinine, salt, and tap water is needed for the continued growth of the mold. Three new batches of the mold were obtained from urine and each placed in a penicillin culture flask containing 100 cc. of the phosphate buffer solution containing creatinine and salt, with the following additions: #1, 300 mg. bactoyeast, #2, 300 mg. bactopectone, and #3, 300 mg. glucose. Only the culture grown on bactoyeast destroyed creatinine on successive transfers to the new creatinine solution. But in other studies, the addition of the following members of the B vitamins separately to the creatinine solution likewise did not give a preparation that would destroy creatinine: thiamin, riboflavin, pyridoxin, nicotinic acid, calcium pantothenate, *p*-aminobenzoic acid, inositol, or choline. Therefore yeast probably contains an additional factor needed for the growth of the mold.

Robbins and Ma (3) stated that biotin was necessary for the growth of 13 different fungi. A new growth of the mold was separated from urine and placed in a penicillin culture flask with 100 cc. phosphate buffer solution containing 100 mg. of creatinine, 100 mg. NaCl, and 0.05 γ biotin (free acid). Luxuriant growth of the mold was obtained, and each of 10 transfers destroyed 100 mg. of creatinine per day when the whole amount of the mold was allowed to stand at room temperature with the creatinine solution.

A batch of active mold was dialyzed against distilled water through a collodion bag in the ice chest. The dialyzate, however, did not destroy creatinine, so that it was impossible to obtain a cell-free dialyzate that would destroy creatinine under these conditions. Growth of the mold was observed as follows on the different substances listed below: creatine, + + +; creatinine, + + + + +; glycine, + + +; glycocyanine, 0, glycocyanidine, 0; arginine, +, and alanine, +. The mold that grows on bread or tomato juice at room temperature did not destroy creatinine under the above conditions. Tyrothricin (Mulford), containing gramicidin and tyrocidin, likewise did not destroy creatinine.

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LETTER TO THE EDITORS

Oxalacetate and the Dephosphorylation of Adenosinetriphosphate

Since Meyerhof *et al.*¹ concluded that the reaction



is irreversible, a number of investigators have studied the mechanism of phosphopyruvate "resynthesis." Kalckar² and Leloir and Muñoz³ have obtained phosphopyruvate formation during the oxidation of four-carbon dicarboxylic acids by tissue; the postulated intermediate being phosphoenoloxalacetate.⁴

In experiments designed to attempt a synthesis of phosphoenoloxalacetate (or phosphopyruvate therefrom), no detectable formation of alkaline-iodine-labile phosphate ester occurred, but with certain enzyme preparations, the addition of oxalacetate was found to cause a loss of ATP phosphorus. Since the results obtained may be of importance to others studying the metabolism of the four-carbon acids, they are presented here. Typical experiments are shown in Tables I and II.

All solutions were neutralized to pH 7.0 before each experiment. Oxalacetate caused a loss of ATP in the presence of an aqueous extract

TABLE I

All tubes contained ATP, inorganic phosphate, MgCl_2 0.005 M, and water to make one ml. Incubated 10 minutes at 25°.

Enzyme ml.	Additions				phosphorus	
	OAA mM	NaF mM	CaCl_2 mM	KCl mM	inorganic μg.	$\Delta 7'$ μg.
.5	.02	.05	.011	.03	125*	73*
	.02				126	70
.5					130	66
.5	.02				196	2
.5		.05			133	68
.5	.02	.05			134	56
.5	.02			.03	185	8
.5			.011		126	71
.5	.02		.011		128	74

* Trichloroacetic acid added before enzyme.

TABLE II

Each tube contained 0.5 ml. enzyme, ATP, inorganic phosphate, $MgCl_2$ 0.005 M, and water to make a final volume of one ml. Incubate 20 minutes at 25°.

OAA mM	Additions		phosphorus	
	NaHCO ₃ mM	pyruvate mM	inorganic μg.	Δ7' μg.
.02	.04	.04	90*	73*
.02			90	71
			167	3
	.04		141	38
		.04	102	60
	.04	.04	156	14

* Trichloroacetic acid added before enzyme.

of ground rat muscle aged (under toluene) until almost free of adenosinetriphosphatase activity. It had no effect in the presence of the aqueous extract of muscle acetone powder. Calcium or fluoride, and, in some experiments 0.01 M malonate, prevented the effect of oxalacetate; oxalate did not.

It is possible that an extremely labile compound is formed by transfer of high energy phosphate to oxalacetate, and that it is hydrolyzed either by enzymes or in the treatment with trichloroacetic acid at 0°.

Pyruvate plus bicarbonate gave more than an additive effect in causing loss of ATP in each of six experiments. The loss increases with time. For example, in one experiment incubation for 10 minutes caused losses of 12, 3, 1, and 8 μg. Δ7' P in the presence of oxalacetate, bicarbonate, pyruvate, and pyruvate plus bicarbonate respectively. At 20 minutes the losses were 27 and 14 μg. Δ7' P for oxalacetate and pyruvate plus bicarbonate respectively. No loss occurred without these additions. The results with bicarbonate and pyruvate indicate the possibility of the Wood and Werkman reaction occurring in muscle.

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Book Reviews

Drying and Dehydration of Foods. By HARRY W. VON LOESECKE, Senior Chemist, Bureau of Agricultural Chemistry and Engineering, Agricultural Research Administration, Agricultural Chemical Division, U. S. Department of Agriculture. Reinhold Publishing Company, New York, N. Y. 302 pp. Price \$4.25.

"Drying and Dehydration of Foods" is a timely, worth while, concise presentation of the latest information on the technology of the dehydration of all kinds of foods.

The first chapter considers the various types of dehydrators now in use in this country. A summary of the methods employed in the drying and dehydrating of fruits in California and in Oregon is next presented. This is not sufficiently detailed to be of much value to a manufacturer. The methods employed in the drying and dehydration of vegetables are next presented. Methods for most of the common vegetables are described. Little is said concerning the quality of the various products.

The dehydration of soups and the preparation of soup mixtures is considered rather briefly, considering the importance of the industry.

A chapter is given to a consideration of the dehydration of eggs, milk and butter. The dehydration of meat, fish and beef blood are described in Chapter 6. The salting and smoking of fish are described briefly, but the presentation of this subject is too sketchy to be of much value. The method described for the salting of fish is that commonly used for cod and haddock, but the text does not indicate that the method described is applicable solely to these fish.

A chapter is devoted to plant sanitation. Another considers the cost of dehydration. The costs presented are largely summaries of data collected by the Dehydration Committee of the Bureau of Agricultural Chemistry and Engineering.

The nutritive value of dried and dehydrated foods is reviewed rather briefly in Chapter 8. A chapter is devoted to packaging and storage. The final chapter presents the methods of analysis and reconstitution of dehydrated foods. A glossary of terms and a patent list round out the book.

Considering the rate at which the dehydration industry is developing and changing, the author has done well to present so much authentic information concerning dehydration and dehydrated foods. Unfortunately, the author will have to rewrite the book approximately once a year if it is to be kept up to date.

DONALD K. TRESSLER, Westport, Conn.

Manometric Methods as Applied to the Measurement of Cell Respiration and Other Processes. By MALCOLM DIXON, Ph.D., Sc.D., F.R.S., University Lecturer in Biochemistry in the University of Cambridge. Second Edition. The Macmillan Company, 60 Fifth Avenue, New York, N. Y., 1943. xiv and 155 pp. Illustrated. 13 × 19.5 cm. Price \$1.75.

This manual of manometric technique is one of those appealingly small, handy and expertly written English classics which stood the test of a decade and longer.

The recent unavailability of the first edition had been felt already and the appearance of a new edition is truly welcome. It probably was wise to keep the character and content of the book practically unchanged. A paragraph on micro methods has been added and, very usefully, the protocols have been included of actual experiments on tissue respiration, glycolysis and respiratory quotient. The emphasis of the book remains on tissue metabolism.

The newer methods and applications might have deserved a more thorough consideration. Emphasis has drifted away from the whole-tissue experiment, when, during the last decade we went more and more into the venture of defining tissue metabolism in terms of isolatable enzyme systems. This change, however, widened rather than lessened the applicability of the manometric method. An amazing variety of reactions emerged that directly or indirectly may be followed gasometrically. Their number is steadily increasing and the manometer remained a dear companion of the worker who once discovered its advantages, although he may have forgotten that he started with tissue slices.

It is true that "*at the present* the ultra-micro methods are rather for the specialist." Nevertheless, a brief acquaintance with the ingenious diver method of Linderström-Lang makes us believe that in the future this method could easily be used as a routine assay method in enzyme isolation work, and may appreciably speed up progress in the field.

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B Vitamins in the Tissues of Rats Maintained at High and Low Temperatures

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INTRODUCTION AND EXPERIMENTAL

In order to throw additional light on the functions of the various B vitamins and their interrelations, and also to ascertain whether temperature may be expected to have an important effect on the composition of meat with respect to its B vitamins, the assays described in this study were performed.

Initially, four groups of rats, three in each group, were reared at Cincinnati; each was killed by a blow upon the head and five tissues from each were packed in dry ice and shipped to Texas where the assays were later performed. The four groups were as follows: (1) kept at 68°F. on a commercial food mixture;¹ (2) kept at 90°F. on the same food mixture; (3) kept at 68°F. on a synthetic diet containing sucrose 76%, casein 18%, salts 4%, Mazola 2%, with the following addenda per kg. of diet: haliver oil 1.2 cc., thiamin 1 mg., riboflavin 4 mg., pyridoxin 4 mg., pantothenic acid 6 mg., inositol 1 g., *p*-aminobenzoic acid 0.3 g., choline 0.75 g., and cystine 2 g.; (4) kept at 90°F. on the same synthetic diet as in (3) except the amount of thiamin was doubled and the choline was increased to 3 g. per kg. of diet. All groups were kept for 3 weeks or more on the diets before being killed.

¹ Wherever "food mixture" or "commercial food mixture" is used in this paper, it refers to the same mixture, namely, Purina Dog Chow.

The enzyme treatment of Cheldelin, *et al.* (1) was used in the preparation of the extracts for the first series of assays for thiamin, riboflavin, niacin, pantothenic acid, folic acid, pyridoxin, and inositol. For the biotin and *p*-aminobenzoic acid assays, for a duplicate set of inositol assays, and for a few pyridoxin assays, acid extracts were prepared as follows. One ml. of 6 *N* HCl was used for each 0.2 g. of tissue, and the digestion was carried out in a sealed tube in the autoclave at 15 lbs. pressure for 2 hours. The contents of the tubes were then evaporated to dryness in a vacuum desiccator and made up to a suitable volume for testing—25–50 mg. tissue per ml. of solution. Exposure of all solutions to light was avoided as far as was feasible.

The assays were carried out in accordance with the methods outlined elsewhere (2). Before the first series of biotin tests had been completed, the tissues had been stored for some months in a dry ice refrigerator, and this may have been partly or wholly responsible for the fact that a remarkable deficiency of biotin appeared in all of the tissues of the rats fed the food mixture, regardless of the temperature of the environment. Since duplicate and triplicate assays on about 50 tissue samples were involved (half on food mixture, half on synthetic diet), we were unable to blame the results on any chance error in handling. In the case of the brains, hearts, and kidneys of the animals maintained on the food mixture, the amount of biotin found was from about 1/50 to about 1/100 that found in parallel tests on the tissues of the rats on the synthetic diets. In the livers and muscles, the contrast in the apparent biotin content between the two sets of animals was less; the tissues from the animals fed the food mixture were 1/5 to 1/20 as rich.

Small amounts of some of the untreated tissues remained at this stage of the study and assays which proved not wholly satisfactory nevertheless showed that when these "deficient" tissues were hydrolyzed *enzymatically* using the method already referred to (1), the apparent biotin content was up to twentyfold greater, whereas the normal tissues extracted in this way gave values in reasonable accord with those obtained by acid hydrolysis.

Because of these unusual results, the biotin assays were repeated upon two entirely new sets of tissues obtained from Cincinnati. The results from the new series of assays are given in Table I, Column 9, and it will be noted that the anomalous contrast does not appear.

In the first set of assays it was apparent on inspection that the

thiamin values for the high temperature animals, Table I, Column 1, were definitely higher than those of the low temperature animals. These assays were carried out using the yeast growth method which has been shown in another study (3) to be not entirely specific. Assays of the fresh sets of tissues for thiamin were therefore run according to the method of Niven and Smiley (4) with some modifications² and the results are given in Table I, Column 2.

p-Aminobenzoic acid assays were carried out using the method of Lewis (5) except that the concentration of the acetate buffer was doubled. This method in our hands has periodically given a great deal of trouble for reasons that are not clear to us. Extensive growth in the blanks often occurs, but this difficulty can be overcome by soaking all the glassware in bromine water, and rinsing thoroughly before use. Individual tests recur which appear to be excellent in all respects, but unfortunately these are not experienced consistently.

DISCUSSION

The unexpected observation with respect to the apparent low biotin content of the stored tissues of animals fed the food mixture cannot readily be explained except on the assumption that the biotin in these tissues is easily destroyed by acid hydrolysis, in comparison with the

² The modifications of the Niven and Smiley method which were used are as follows: All glassware was cleaned with dichromate cleaning solution, rinsed 5 times with tap water and 3 times with distilled water. Sodium thioglycolate was sterilized and was introduced aseptically as a separate addendum. In the preparation of the yeast extract supplement, before final adjustment to 200 ml. volume, the solution was shaken with 5 g. of activated charcoal (Darco G-60), filtered, and the pH readjusted to 7.4. The procedure of sterilizing, introducing solutions, and inoculating was modified thus: The standards and sample extracts, 0.01–0.1 ml. portions, were pipetted into thoroughly clean test tubes, and 0.4 ml. of sodium acetate buffer, pH 4.5, was added to each tube. Tubes, medium, solution of sodium thioglycolate (10 mg./ml.), and all pipettes to be used were autoclaved at 15 lbs. pressure for 15 minutes. After cooling to room temperature, the medium was inoculated with a suspension of *Streptococcus salivarius* (prepared according to Niven and Smiley), one drop for each 10 ml. of medium, and the flask of inoculated medium was carefully but thoroughly shaken. (Substances which precipitated out of the medium on autoclaving redissolve almost entirely on shaking.) Ten ml. of the inoculated medium and 0.1 ml. of the sodium thioglycolate solution were pipetted aseptically into each tube. The test was incubated at 37°C. for approximately 24 hours, although maximum growth is usually obtained within 16–18 hours.

TABLE I

*B Vitamins in Tissues of Rats Maintained at Low and High Temperatures**

(γ/g., dry weight)

Thiamin		Ribo- flavin	Nia- cin	Panto- thenic Acid	Folic Acid	Pyri- doxin	Biotin		Inositol		<i>p</i> - Amino- benzoic Acid
	Set No. 2†							Set No. 2†	Acid Extr.	Enz. Extr.	
Brain, Low Temp., Food Mixture											
17.1	10.7	17.9	186	62.0	5.5	0.5	0.01	.39	6100	6730	.079
16.0	10.1	19.8	254	83.2	10.0	1.26	0.02	.29	5810	6150	<.1
22.8	9.3	16.8	253	67.4	6.0	1.92	<0.01	.25	4850	5100	.032
Brain, High Temp., Food Mixture											
21.8	8.8	21.0	235	73.4	7.5	1.11	<0.02	.31	6600	5730	.089
23.2	11.4	16.0	266	63.0	8.7	1.02	<0.02	.23	4900	3830	.050
21.0	10.1	21.8	272	77.8	13.1	2.6	<0.02	.20	4510	4350	—
Brain, Low Temp., Synthetic											
10.6	—	10.0	202	56.4	9.6	1.76	0.43	—	3840	3760	.188
13.8	—	11.3	214	57.5	7.68	1.81	0.83	—	3100	5360	.063
13.0	—	13.5	192	58.3	7.6	1.88	0.85	—	5950	6180	.049
Brain, High Temp., Synthetic											
19.2	—	18.1	218	68.8	6.3	1.52	1.35	—	4540	7770	.053
9.9	—	16.7	208	60.2	12.4	1.73	0.73	—	5650	5810	.083
14.0	—	19.3	228	62.8	9.45	1.2	0.98	—	3880	5410	.065
Heart, Low Temp., Food Mixture‡											
25.6	17.0	69.6	420	127	6.15	1.28	0.03	2.06	1550	729	.091

* Each series of values is listed in the same order; *e.g.*, the first values on Brain, Low Temp., Food Mixture, apply to the same animal as the first values on Liver, Low Temp., Food Mixture.

† Different set of rats (same for thiamin and biotin). See text.

‡ Values from assays on composite samples from three animals, except thiamin and biotin values on Set No. 2, which show average values from assays on separate samples from three animals.

TABLE I—Continued

Thiamin		Ribo- flavin	Nia- cin	Panto- thenic Acid	Folic Acid	Pyri- doxin	Biotin		Inositol		<i>p</i> - Amino- benzoic Acid
	Set No. 2†							Set No. 2†	Acid Extr.	Enz. Extr.	
Heart, High Temp., Food Mixture‡											
37.9	18.2	60.5	436	146	5.05	1.9	0.03	.94	1390	503	.074
Heart, Low Temp., Synthetic‡											
14.3	—	69.3	417	124	5.02	1.13	2.42	—	809	358	.318
Heart, High Temp., Synthetic‡											
29.6	—	68.0	350	113	5.37	1.75	2.31	—	1120	1061	.164
Kidney, Low Temp., Food Mixture											
18.9	13.3	157	388	158	17.4	1.71	0.04	2.73	3960	4200	.209
26.2	7.4	151	503	219	25.0	1.55	0.06	1.89	3490	4770	.273
28.7	12.0	167	443	259	30.0	1.53	0.07	3.28	3020	4970	.216
Kidney, High Temp., Food Mixture											
44.1	12.5	131	439	201	17.7	5.0	0.06	3.56	3100	3580	.079
48.0	10.8	125	420	189	18.5	5.1	0.03	2.43	2380	2640	.311
29.4	7.6	132	444	182	17.0	4.9	0.07	2.22	3120	3020	.179
Kidney, Low Temp., Synthetic											
10.0	—	123	368	139	29.2	1.87	5.41	—	2520	2580	.87
8.2	—	119	374	152	41.1	1.63	5.60	—	2440	2700	.109
9.5	—	139	411	166	38.0	3.0	6.96	—	4230	3980	1.91
Kidney, High Temp., Synthetic											
11.9	—	173	348	167	19.4	2.62	5.59	—	4130	5260	1.13
13.0	—	165	368	139	18.5	3.58	4.56	—	3720	4310	1.09
10.6	—	143	354	121	19.8	2.56	5.95	—	3760	3270	1.00

TABLE I—*Continued*

Thiamin		Ribo- flavin	Nia- cin	Panto- thenic Acid	Folic Acid	Pyri- doxin	Biotin		Inositol		<i>p</i> - Amino- benzoic Acid
	Set No. 2†							Set No. 2†	Acid Extr.	Enz. Extr.	
Liver, Low Temp., Food Mixture											
43.6	13.9	117	555	429	34.4	2.62	0.67	1.29	1930	1200	.307
46.4	12.5	121	515	445	28.6	2.06	0.49	2.51	2060	940	.229
39.6	17.0	116	520	457	29.4	2.68	0.11	1.44	1920	1280	.144
Liver, High Temp., Food Mixture											
61.5	15.7	76.4	487	346	35.1	1.39	0.70	1.29	1970	514	.237
54.0	18.5	79	474	451	23.2	1.79	0.34	1.5	1820	1120	.152
52.5	21.1	91	490	333	29.8	1.9	0.51	1.55	1820	660	.162
Liver, Low Temp., Synthetic											
15.4	—	84.0	499	226	50.8	1.74	3.78	—	2200	658	.848
13.6	—	92.4	563	206	110	2.98	3.81	—	1700	492	.126
10.2	—	77.7	424	153	60.5	2.34	3.05	—	1150	667	.54
Liver, High Temp., Synthetic											
18.1	—	93.3	385	160	42.0	2.13	—	—	—	1540	—
14.3	—	89.0	236	278	59.5	2.56	2.63	—	2750	660	1.00
14.2	—	87.7	480	177	60.0	2.52	4.83	—	2030	733	.423
Muscle, Low Temp., Food Mixture											
10.3	1.6	13.1	292	26.6	1.81	1.39	0.01	.17	778	267	.067
—	1.6	—	—	—	—	—	—	.13	—	—	—
10.4	2.0	12.7	290	30.9	2.67	0.82	0.01	.15	1110	483	.059
Muscle, High Temp., Food Mixture											
13.7	2.4	10.9	314	37.4	3.52	4.0	0.02	.12	838	396	.078
14.4	1.4	10.6	302	28.2	2.7	0.9	0.01	.16	1160	718	.018
12.5	2.1	10.1	282	30.7	2.8	5.7	0.01	.13	900	241	.1

TABLE I—*Continued*

Thiamin		Ribo- flavin	Nia- cin	Panto- thenic Acid	Folic Acid	Pyri- doxin	Biotin		Inositol		<i>p</i> - Amino- benzoic Acid
	Set No. 2†							Set No. 2†	Acid Extr.	Enz. Extr.	
Muscle, Low Temp., Synthetic											
—	—	—	—	—	—	—	—	—	—	—	—
4.7	—	7.8	312	45.8	4.55	2.52	0.47	—	480	408	.216
5.7	—	8.3	360	52.3	4.52	4.42	0.31	—	386	314	.151
Muscle, High Temp., Synthetic											
3.3	—	7.4	294	36.1	3.46	3.46	0.27	—	1030	370	.187
4.4	—	7.2	238	40.0	4.04	2.60	0.28	—	652	199	.163
6.5	—	8.8	364	45.7	5.44	2.81	0.07	—	707	302	.106

biotin of animals fed on a "synthetic" diet. Using relatively fresh tissues, the experimental findings could not be duplicated.

The only cases in which there seems a consistent difference between the tissues from the low temperature and high temperature animals are those thiamin assays made by the yeast growth method. When the assays were repeated using the *S. salivarius* method, the two sets of tissues appeared to agree remarkably well.

While the presence of additional thiamin in tissues of the high temperature animals does not seem to be demonstrated or indicated, the presence of larger amounts of some unknown yeast growth stimulating substance does seem to be demonstrated. If we accept the thiamin values as determined by the *S. salivarius* method as reliable, then the unknown yeast growth stimulant appears to be largely absent from the low temperature synthetic-diet animals, because the yeast growth method gave values in four tissues, excluding muscle, in good agreement with those obtained by the *S. salivarius* method. The unknown yeast growth stimulant appears to be particularly abundant in the tissues of rats kept at high temperature on the food mixture.

So far as the known B vitamins are concerned, it appears that no significant differences in content between rats in cold and warm environments have been demonstrated. If such differences exist, larger numbers of animals must be studied in order to demonstrate them.

It appears that, in connection with the diets used in this study, temperature is not an important factor in causing variation in the B vitamin content of tissues. The existence of an unknown yeast growth stimulant in greater concentration in the tissues of rats kept in warm environment is strongly indicated.

Insofar as it is safe to carry over observations on one animal to others, it seems probable that meat obtained from animals raised at higher or lower temperatures (provided temperatures were the only variable) would probably not differ materially with respect to the content of known B vitamins.

A comparison of the inositol values obtained by acid and enzyme digestion suggests that inositol is not in the same combination in all of the tissues. In brain and kidney, for example, the two methods of extraction yield about the same results. In heart, liver, and muscle the amount obtained by enzymatic digestion was consistently lower, sometimes less than $\frac{1}{3}$ as great as that obtained by acid hydrolysis.

SUMMARY

Under the conditions studied, temperature does not appear to be an important factor in causing variation in the B vitamin content of rat tissues.

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Studies on Penicillinase

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INTRODUCTION

In the production of penicillin, bacterial contamination of the mold fermentation will often result in the complete destruction of the penicillin formed. Several bacteria isolated from such mold fermentations have been investigated, and studies on the inhibitor (penicillinase) produced are here reported.

Abraham and Chain (1) first described the penicillin inhibitor from bacteria. Because of its enzymatic properties, they named it penicillinase. They demonstrated the production of the enzyme by *Bacterium coli*, *Micrococcus lysodeikticus*, and a Gram-negative rod found as a contaminate of some *Penicillium* cultures. Abraham, *et al.* (2) were unable to detect the enzyme in cells of a *Staphylococcus aureus* made insensitive to penicillin. Harper (4) prepared extracts of paracolon bacilli which had greater penicillin destroying action than similar preparations from *Bact. coli*. Bondi and Dietz (3) showed that coliform bacilli, aerobic sporeforming bacilli, and certain strains of *Shigella* produced the penicillin inhibitor. Although Himes and White (5) reported that bacteria were the only organisms of those they tested capable of producing penicillinase, Woodruff and Foster (9) reported that besides bacteria, actinomycetes, fungi, and yeast also produced the enzyme in varying amounts. Kirby (6) made preparations from seven "naturally" penicillin-resistant strains of *Staph. aureus* which had penicillinase activity, while similar extracts of penicillin-sensitive strains of *Staph. aureus* had no activity.

METHODS

Assay for penicillin. The cup-plate method (8).

Assay for penicillinase. 0 to 5 ml. samples of the enzyme in water of pH 7.0 buffer are pipetted into 18 × 150 mm. test tubes. Enough 1% pH 7.0 phosphate buffer is added to each to bring the total volume to 10 ml. Into each tube 1 ml. of a standard penicillin solution containing 50 Oxford units per ml. is pipetted, well mixed, and

then incubated 1 hour at 37°C. After incubation the tubes are placed in an ice-bath and assayed for penicillin. The values are read from a standard curve constructed as described below.

A standard curve is constructed by assaying a suitable solution of penicillinase at 5 to 10 different levels. The percent recovery of penicillin for each tube (as calculated for a control tube with no enzyme) is plotted against amount of enzyme. The ordinate is arbitrarily divided by 5 equispaced points which are called penicillinase units 1 to 5. On our curve Fig. 1, one unit shows a 42.5% recovery of penicillin.

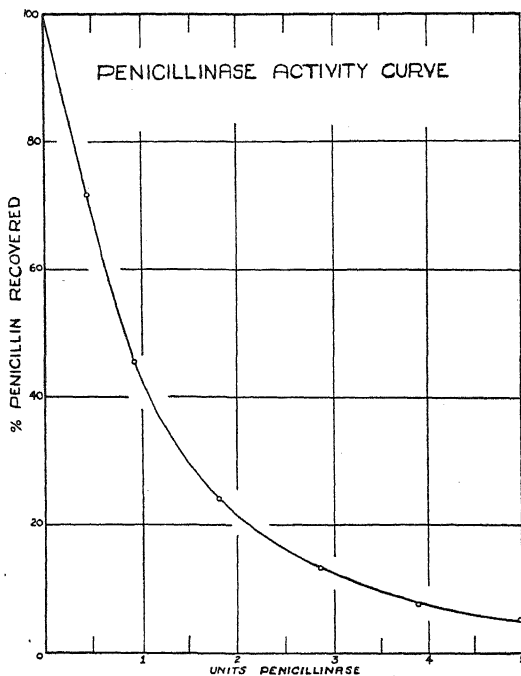


FIG. 1

Thus for present convenience a unit of penicillinase is that amount of enzyme which, in 11 ml. of pH 7.0 solution containing 50 Oxford units of penicillin, will destroy in 1 hour at 37°C. an amount of penicillin equal to 57.5% of the penicillin recovered in the control.

Preparation of Bacterial Cells. All bacteria were grown in a medium of 1% glucose, 0.5% peptone (Difco), 0.1% yeast extract (Difco), 0.05% KH_2PO_4 , 0.05% K_2HPO_4 , and 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. 500 ml. sterile portions of medium were inoculated with a loopful of cells from stock agar slants and incubated 24 hours at 30°C. These were then used to inoculate 13 l. quantities of medium in 5 gal. carboys which were incu-

bated 48 hours at room temperature. The bacterial cells were harvested with a Sharples supercentrifuge and immediately lyophilized. The yield of dry cells from 13 l. of medium was from 1.0–1.3 grams.

Extraction of Enzyme. Plasmolysis, autolyses at 30°C., and buffer extractions were all tried for liberation of the penicillinase. Buffer extraction as described was found most satisfactory. Although the method is called a buffer extraction, autolysis undoubtedly takes place to a large extent and is responsible for the liberation of much of the enzyme.

5 g. of lyophilized cells are mixed for 1½ to 2 hours in a Waring type blender with ice cold, 1%, pH 7.0 phosphate buffer. The mixture is cooled every 15 to 20 minutes during this procedure. After mixing, the cell suspension is kept in the refrigerator at 5–10°C. for 2 days with occasional stirring. After refrigeration the suspension is centrifuged in the cold (7) for 1 hour at 3000 r.p.m. The centrifugate is decanted and recentrifuged cold for another hour. The final centrifugate is filtered through a fine fritted glass filter (not an asbestos type filter) and stored in the refrigerator. This concentrate which is used for further work has up to 700 units of penicillinase per ml.; it is stable in the refrigerator for several weeks.

EXPERIMENTAL

Three bacteria were isolated from *Penicillium notatum* fermentations which had been infected and showed no penicillin activity. The bacteria were labelled PD 1, 2 and 3 (PD = penicillin destroying). All were very small Gram-negative rods, PD-2 was a spore former. Cultures were kept on nutrient-agar slants and transferred at frequent intervals.

Effect of Medium on Enzyme Production

A series of fermentations using the bacterium PD-3 were set up in which various media were used and the penicillinase activities of the bacterial cells compared. The regular medium was altered in the following ways:

1. The glucose removed and the peptone increased to 1%.
2. The glucose replaced by lactose.
3. The glucose replaced by sucrose.
4. The yeast extract replaced by malt extract.
5. The glucose increased to 2%.

The fermentation without added glucose (no. 1) was slightly superior to all others including the one with the regular medium. However, the cells so obtained were so undesirable to work with that this fermentation was not used. The regular medium was superior to all

others. Malt extract could not replace yeast extract, nor could sucrose or lactose efficiently replace glucose.

Aeration experiments were run on PD-3 fermentations with the regular medium and the 1% peptone medium. 1 l. quantities were aerated for 24 hours at 30°C., the cells harvested, extracted, and the extracts assayed for penicillinase. Aeration slightly increased the enzyme yield for the peptone fermentation and slightly decreased the yield for the regular fermentation.

Precipitations

I. Experiments with extracts from PD-2 cells. The enzyme in extracts of PD-2 cells is precipitated quantitatively in a saturated $(\text{NH}_4)_2\text{SO}_4$ solution, a 50% acetone or dioxane solution, an acid sodium tungstate solution, and in a 60% ethanol solution. A 43% acetone solution and a half-saturated $(\text{NH}_4)_2\text{SO}_4$ solution while precipitating much material from the cell extract only precipitate about 10% of the enzyme. When more purified extracts of penicillinase were precipitated with acetone, a huge loss of activity was obtained. The effect of acetone on such preparations is reported below.

II. Experiments with extracts from PD-3 cells. The extracts from lyophilized PD-3 cells are from 5 to 10 times as potent in penicillinase activity as those from PD-2 cells. With these extracts, solvent precipitations (acetone, dioxane, alcohol) became very variable. While apparently all the activity could be precipitated, the recoveries were very small in almost all cases. Very occasionally good recoveries were obtained by acetone precipitation. This variation is being studied further. $(\text{NH}_4)_2\text{SO}_4$ precipitations were the same for both PD-2 and PD-3 cell extracts. With PD-3 extracts, $(\text{NH}_4)_2\text{SO}_4$ had an apparent stimulatory effect. Preparations after $(\text{NH}_4)_2\text{SO}_4$ precipitation would assay greater potency (10–20% greater) than the starting material. Part of this effect might have been due to the removal of inhibitory substance from the extracts. After $(\text{NH}_4)_2\text{SO}_4$ precipitation, the redissolved precipitate was non-dialysable. However, there was a slow decrease in activity, even with dialysis at 5°C. That this loss is not due to dialysis of either the enzyme or a possible coenzyme was shown by assaying both the dialyzate, the water surrounding the cellophane dialysis bag, and the combination of the two. The water around the bag showed no activity, and the addition of this solution to some of the dialyzate gave no increase in the activity over the dialyzate alone.

Heat Inactivation

The penicillinase is very readily inactivated by heat. At 45°C. a pH 7.0 solution of enzyme containing 20 units per ml. lost 66% of its activity in 20 minutes and over 95% in one hour. A similar solution kept at 5°C. was stable for several days.

Effect of pH

A series of tubes were set up in a manner similar to that used in the assay method for penicillinase. A buffer of different pH was used for

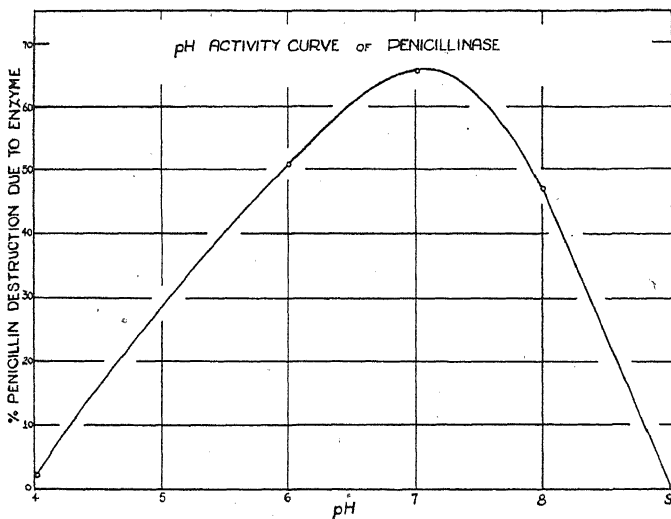


FIG. 2

each series of two tubes (one with a constant amount of enzyme and one with no enzyme). The tubes were incubated one hour at 37°C. and immediately cooled in an ice-bath. They were adjusted to pH 7.0, diluted, and assayed for penicillin. Fig. 2 shows the pH activity curve obtained by plotting the per cent penicillin destruction due to the enzyme against the pH. The "optimum" pH for the enzyme at 37°C. was found to be pH 7.1.

Effect of Reagents

The enzyme is inhibited by iodoacetic acid, amyl acetate, and acetone; and dioxane when concentrated preparations are studied. Sodium

fluoride and sodium cyanide have no effect on the penicillinase. Under certain conditions *dl*-phenylalanine has a marked stimulating effect, indole-3-acetic acid has a moderate inhibiting effect (up to 50% inhibition).

Adsorption and Elutions

I. Experiments with extracts from PD-2 cells. It was found early in the investigation that enzyme preparations filtered through a Seitz filter lost all their activity. Because the enzyme could be filtered through ceramic type bacterial filters, it was assumed that the penicillinase was adsorbed by the asbestos of the filter pad. To prove this assumption, acid washed asbestos for Gooch crucibles was tested as an adsorbent. At pH 7.0, 1 g. of asbestos adsorbed all the enzyme in 50 ml. of a solution containing 50 units of penicillinase. Decalso, magnesia, Florisil, Super Filtrol, Special Filtrol, Fisher's adsorption alumina, zirconium oxide, and Hyflo Super Cell were all tested for their adsorption properties by mixing the adsorbent in a pH 7.0 solution of the enzyme, removing the adsorbent by centrifugation, and assaying the centrifugate for penicillinase activity. Hyflo, zirconium oxide, magnesia, Florisil and the Filtrols were all strong adsorbents; alumina was fair, and Decalso was very weak. On investigating Hyflo and asbestos further, Hyflo was found to be the stronger adsorbent and the more convenient with which to work. The following method was devised to use a Hyflo adsorption and elution as a purification step.

A pH 7.0 solution of the enzyme containing 250 units per 50 ml. was stirred 30 minutes in an ice-bath with 1 g. of Hyflo. The Hyflo was removed by centrifuging and the enzyme eluted by mixing 45 minutes in an ice-bath with 50 ml. of 0.8 *N* NH_4OH . A 90% recovery of the penicillinase with a 4-5 times concentration was effected.

II. Experiments with extracts from PD-3 cells. Using the same technique as described above for PD-2 extracts, a comparable adsorption was obtained, but only 10-30% of the activity was recovered after elution. We believe this loss was due to destruction (instability) because a second elution yielded no appreciable increase in activity.

Alkali Extraction

Solubility studies on an acetone precipitate of a PD-2 cell extract showed the enzyme to be insoluble and stable in cold *N*/10 NaOH. Thus by mixing some of the acetone precipitated material in *N*/10

NaOH, a large amount (50–60%) of the impurities was dissolved, and the enzyme remained insoluble and was filtered out. Because of the inability to obtain an acetone precipitate of the extract from PD-3 cells, this method of purification could not be used for PD-3 extracts.

Penicillinase Producing Ability of PD Bacteria and Their Sensitivity to Penicillin

In our work with PD cultures we have noted a large variation in the ability of different bacteria to produce penicillinase. Also a PD-2 culture which had been kept on the same agar slant for several months lost much of its ability to produce the enzyme. It was thought desirable to see if there was any correlation between enzyme production and penicillin sensitivity for these bacteria.

The penicillinase producing ability of the bacteria was determined using a slight modification of the method given for preparing cells and extracting the enzyme.

The sensitivity of the organism to penicillin was determined by growing the bacteria in 10 ml. portions of the medium described above which had either no penicillin, 100 Oxford units, or 200 Oxford units. Each series of three tubes was inoculated with a drop of dilute bacterial suspension (5 drops of a 24 hour culture diluted with 10 ml. sterile saline) and incubated 18 hours at 30°C. The turbidity of the tubes was determined with a Luxtrol colorimeter. When there was no effect from penicillin, there was no difference in turbidity. Table I shows the relative abilities of the PD cultures to produce penicillinase and their sensitivity to penicillin.

Action of Penicillinase on the Penicillin Produced by Two Strains of Penicillium notatum

The penicillin produced by strains NRRL-1249-B21 and NRRL-832 of *Penicillium notatum* were tested for their sensitivity to a penicillinase

TABLE I
Relative Penicillinase Production and Penicillin Sensitivity of PD Bacteria

Culture of Bacteria	Relative Production of Penicillinase	Turbidimetric Differences	
		100 units Penicillin	200 units Penicillin
PD—1	22	16	23
PD—2	11	24	51
PD—2 (old)	6	32	53
PD—3	60	0	0

preparation. Using the method described for penicillinase estimation, it was found that both penicillins were equally destroyed by penicillinase.

The fact that the penicillin from strain NRRL-1249-B21 was destroyed to a larger extent (about 50%) than that from strain NRRL-832 during the one hour incubation, points to the possible assumption that at least two types of penicillin were present.

DISCUSSION

The properties of the penicillin inhibitor obtained from the PD cultures described are on the whole consistent with those described by other workers for similar preparations from other bacterial sources (1, 2, 3, 4, 5, 6, 9). Because the inhibitor is heat labile (1), precipitated by protein precipitants (1, 4, 6), non-dialyzable (1), and has an "optimum" pH, it has been considered an enzyme and called penicillinase. Abraham and Chain (1) found an "optimum" pH of 8 to 9. For our preparations we find the greatest activity at pH 7.1 for 37°C.

The enzyme is extremely labile, especially in more purified solutions. It is 66% destroyed in 20 minutes at 45°C., is inactivated by acetone when this solvent is used as a precipitating agent for purified enzyme solutions. We found an inhibition of the enzyme by iodoacetic acid as first reported by Woodruff and Foster (9), but could not get a stimulatory action from thioglycolate as they reported for their preparations. We do get a stimulatory effect with *dl*-phenylalanine, and an inhibitory effect with indole-3-acetic acid under some conditions.

There is apparently no rigid relationship between the ability of the bacteria to produce penicillinase and their sensitivity to penicillin (1, 2, 9). However, with our PD cultures, a definite correlation exists. The best enzyme producer, PD-3, was not inhibited in growth by 200 Oxford units of penicillin in 10 ml. of medium, while the poorer producers, PD 1 and 2, were definitely inhibited by this concentration of penicillin. Moreover, the smaller the ability of the culture to produce penicillinase the more it was inhibited in growth by penicillin.

SUMMARY

1. A method is given for the estimation of penicillinase activity and a penicillinase unit defined.
2. A method of extracting the enzyme from bacterial cells is described.

3. Various media were investigated for use in growing the bacteria for penicillinase production. A 1% peptone medium without added sugar gave the highest yield of enzyme, but a $\frac{1}{2}$ % peptone, 1% glucose medium produced the most desirable working material. Glucose could not be replaced efficiently by either sucrose or lactose in this medium.

4. Acetone, alcohol, dioxane, sodium tungstate, and saturated $(\text{NH}_4)_2\text{SO}_4$ solution precipitate the enzyme. Acetone, alcohol, and dioxane inhibit the penicillinase when purified enzyme solutions are used. The enzyme is non-dialyzable through cellophane membrane.

5. Penicillinase in purified solution is extremely labile. It is 66% destroyed at 45°C. in 20 minutes, over 95% destroyed in an hour.

6. The most active pH at 37°C. is pH 7.1.

7. The enzyme is inhibited by iodoacetic acid, amyl acetate, and partially by indole-3-acetic acid. It is activated by *dl*-phenylalanine.

8. Adsorption studies are given along with a purification method using adsorption and elution.

9. Correlation is shown between the ability of PD bacteria to produce penicillinase and their sensitivity to penicillin.

10. A penicillinase preparation is shown to have the same destructive action on the penicillin produced from *Penicillium notatum* strains NRRL-1249-B21 and NRRL-832.

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The Effects of Plant Growth Substances Upon the Metabolism of Yeast *

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INTRODUCTION

A considerable amount of work has been reported concerning the effects of the plant hormones upon the development of plant tissue. The subject has been recently reviewed by Zimmerman (1, 2). Although little is known about the specific mechanism by which these substances gain their results, there is some evidence that they may influence physical properties such as protoplasmic streaming (7), or chromosome structure (8), or the mobilization and transport of food-stuffs (9). An enzymatic approach to the problem has been followed by Commoner and Thimann (4) and by Berger and Avery (10). There is only scanty knowledge available on the metabolic processes of plants in general, and no such scheme as the Embden-Meyerhof theory for carbohydrate metabolism exists in the case of plants. However, a study of the isolated investigations reported (3, 4, 5, 6) leads to the conclusion that some corresponding series of reactions is operative in the metabolism of plants; and while there may be differences in specific detail it seems a reasonable assumption that the general plan is similar in plant and animal cells. If this assumption is valid the reasoning advanced by Potter (16, 17) to explain normal and abnormal growth in animal tissues may apply to plant tissue as well. This application may be stated briefly, as follows: There are present in tissue two types of cells, mature and undifferentiated. The mature types are fully differentiated and are concerned primarily in the performance of a specific function. The undifferentiated cells, represented in animal

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tissue by fibroblasts and tumor cells, are concerned primarily with growth. The mature cell gains most of its energy from aerobic processes and requires oxygen in order to live. The undifferentiated cell, on the other hand, gains a large share of its energy from anaerobic processes and can survive well under anaerobic conditions. There is, under natural conditions, a constant competition between the two types of cells for a limited supply of energy-yielding and structural materials. Usually, the mature cell is the better competitor, and consequently the undifferentiated cell's growth is restricted. However, when the environment is changed in such a manner as to allow the undifferentiated cell to compete favorably with the mature cell, growth results. This change in the conditions of competition may be brought about in a number of ways: (1) the supply of raw materials may be made sufficiently large so as to furnish enough for both types of cells; (2) the balance of numbers of the two types of cells may be changed by destroying one type preferentially, or by adding one type, as in a tumor transplant or by causing the formation of one type as in hydrocarbon application or virus injection; and (3) environmental conditions may be so adjusted as to favor the survival of one type of cell. Such an adjustment might be made locally by the introduction of certain chemical substances.

The plant growth substances appear to belong under point (3). To elucidate the mechanism of action of these compounds a study of their effects upon specific metabolic systems was instituted. Data have been obtained which indicate that at certain concentrations these chemicals inhibit preferentially the enzymes concerned with the aerobic phase of metabolism.

EXPERIMENTAL

Yeast was chosen as experimental material because the details of its metabolism are comparatively well known, it is convenient and easy to handle, and it is readily obtainable. The yeast used in all experiments was Red Star commercial compressed yeast obtained through a local retail outlet. It was stored in the cold in a covered container to prevent moisture loss. A 5 per cent suspension of this product was dispersed in cold, distilled water just before use with the aid of a hand-operated Potter-Elvehjem homogenizer (11). One-tenth of a ml. per flask was used routinely.

The basic medium used in aerobic experiments was a phosphate buffer consisting of a 1-1 mixture of 0.15 *M* NaH_2PO_4 plus NaOH to pH 6.5 and 0.15 *M* KH_2PO_4 plus KOH to pH 6.5. One ml. of this buffer was added to a reaction mixture of total volume of 3.0 ml. giving a final molarity of 0.05 as recommended by Potter (12). In anaerobic experiments 0.1 *M* NaHCO_3 and a gas mixture of 30% CO_2 + 70% N_2

was used as a buffer giving a pH of 6.5. Substrates and inhibitors were added as aqueous solutions of the sodium salts or hydrochlorides of appropriate concentration and adjusted to pH 6.5 where necessary. The *p*-phenylenediamine, dimethyl-*p*-phenylenediamine, quinones, and indole propionic acid used in these experiments were Eastman products. We are indebted to Dr. E. H. Volwiler of the Abbott Laboratories for generous supplies of the plant growth substances. Dimethyl-*p*-phenylenediamine and *p*-phenylenediamine were tested because they are split products of a carcinogenic azo dye and a comparison between their action and that of the plant growth substances would therefore be of interest. Quinone and naphthoquinone were used because some of their effects on other enzyme systems are known.

Manometric measurements were carried out in a Warburg apparatus of conven-

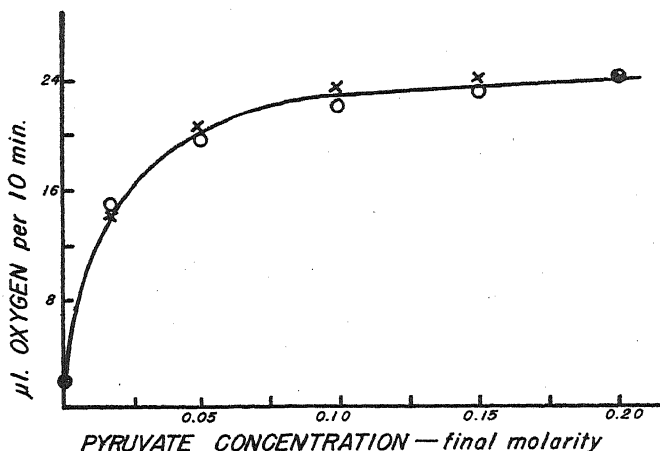


FIG. 1

The Effect of Pyruvate Concentration upon Its Oxidation by Yeast
Checks of duplicates indicated by circles and crosses.

tional design at a temperature of 38°C. using flasks with or without side arms as indicated by the nature of the experiment.

Pyruvic Acid Oxidation. It was thought that the enzymes which lead to pyruvate utilization would probably be the most sensitive to inhibitors, therefore, they were studied first. Pyruvic acid used as a substrate was redistilled at reduced pressure from an original Eastman supply and immediately diluted and standardized at 1.00 *N*. This stock solution was stored in the cold room and from it suitable working solutions were prepared as required. Neutralization to pH 6.5 was carried out just before use to avoid formation of toxic polymers, and care was taken not to overrun the end-point; an addition of a small amount of buffer toward the end of the titration was particularly helpful in this regard. Preliminary investigations showed that 0.6 ml. of 0.05 *M* pyruvate per flask provided enough substrate to saturate 0.1 ml. of 5% yeast suspension during the 30 to 60 minute experimental period (Fig. 1). Graded

concentrations of the inhibitors to be studied were then added to the system and the rate of oxygen uptake measured. In all cases the inhibitor was incubated with the yeast for fifteen minutes at room temperature before addition of substrate which was added just before placing the flasks in the thermostat.

Glucose Oxidation. The next system selected for study was the aerobic utilization of glucose by yeast. The same procedure was followed as above except that 0.6 ml. of 0.25 *M* glucose was substituted for the pyruvate. Glucose was Merck's reagent grade dextrose made up into solutions of suitable concentration immediately before use.

Anaerobic Glucose Utilization. In this series of experiments a bicarbonate-carbon-dioxide buffer, as described previously, was substituted for the phosphate buffer and yellow phosphorus was placed in the center cups to insure anaerobic conditions. Carbon dioxide evolution was measured and the effects of the various inhibitors thus followed.

TABLE I
The Recovery of Lactic Acid in the Presence of Inhibitors

Compound	Final Molarity of Inhibitor	Average Per Cent Recovery of Added Lactic Acid*
Naphthoquinone	0.000015	98.0
Quinone	0.00015	98.0
<i>p</i> -Phenylenediamine	0.0004	98.0
Dimethyl- <i>p</i> -phenylenediamine	0.0004	108.0
Naphthoxy heptanoate†	0.005	94.0
Naphthyl acetate†	0.020	102.0
Indole butyrate†	0.025	103.0

* Values are the average of determinations on 3.0 ml. of duplicate reaction mixtures (see text) containing 60 and 120 μ g. of added lactic acid respectively.

† Sodium salts.

Lactic Acid Production. The experiments in this series were designed to delimit more closely the locus of action of the inhibitors. They were carried out in the same manner as the aerobic glucose utilization experiments except that at the end of each run 0.5 ml. of 50% trichloroacetic acid was added to each flask, the contents transferred to centrifuge tubes and the precipitate removed by centrifuging. Suitable aliquots of the supernatant were then taken for further analysis. Lactic acid was determined by the method of Barker and Summerson (13) as modified by LePage and Umbreit (14). The runs in which *p*-phenylenediamine and dimethyl-*p*-phenylenediamine were used required that blanks be run to correct for the colors of the amines. The other compounds studied gave no interference.

It was necessary to determine whether lactic acid was being recovered quantitatively in the presence of inhibitors. In order to check this point, buffer, inhibitors at appropriate molarities, standard lactic acid at two levels (60 and 120 μ g. in tubes otherwise duplicates), and water to give a volume of 3.0 ml. were added to tubes and the mixture incubated 45 minutes at 38°C. with occasional shaking. At the end

of this time 0.5 ml. of 50% trichloroacetic acid was added to each tube, the tubes centrifuged where necessary, and lactic acid determined in the usual manner upon a suitable aliquot of the supernatant. Recoveries listed in Table I show that the presence of the inhibitors did not affect the lactic acid determination.

Ethyl Alcohol Production. In addition to measuring lactic acid on the above trichloroacetic filtrates, ethyl alcohol was also determined. The method of Kozelka and Hine (15) was used with slight modifications to accommodate lower concentrations of alcohol.

RESULTS

An interesting finding is shown in Table II. Here it will be noted that the compounds are arranged according to the parent molecules for which enough homologues were available to make such a study.

TABLE II
Correlation between Length of Side-chain and Inhibitor Effectiveness among Various Groups of Inhibitors

Compound*	Final Molarity at 100 per cent Inhibition of Pyruvate Oxidation
Naphthoxy Group:	
2-Naphthoxy heptanoate	9.0×10^{-3}
2-Naphthoxy valerate	18.0×10^{-3}
1-Naphthoxy propionate	31.0×10^{-3}
2-Naphthoxy acetate	90.0×10^{-3}
1-Naphthoxy acetate	90.0×10^{-3}
Naphthyl Group:	
1-Naphthyl butyrate	21.0×10^{-3}
1-Naphthyl propionate	40.0×10^{-3}
1-Naphthyl acetate	40.0×10^{-3}
Indole Group:	
Indole butyrate	40.0×10^{-3}
Indole propionate	80.0×10^{-3}
Indole acetate	200.0×10^{-3}

* Sodium salts.

It can be seen from Table III that four of the compounds studied were definitely better inhibitors of pyruvate oxidation than of glucose oxidation. However, in the case of naphthoxy valerate, naphthoxy heptanoate, and naphthoxy propionate differences were small at levels which gave 100% inhibition of pyruvate oxidation. Nevertheless, Fig. 2 which illustrates the effect of naphthoxy heptanoate demonstrates that a difference does exist even in the case of these highly effective inhibi-

TABLE III

Comparison of the Effect of Inhibitors upon Pyruvate and Glucose Oxidation by Yeast

Compound	Per Cent Inhibition of Pyruvate Oxidation	Per Cent Inhibition of Glucose Oxidation
1-Naphthoxy valerate (0.007 M)*	100	100
2-Naphthoxy heptanoate (0.009 M)*	100	93
1-Naphthoxy propionate (0.030 M)*	100	95
Naphthyl acetate (0.045 M)*	100	86
Indole butyrate (0.040 M)*	100	76
<i>p</i> -Chlorophenoxy acetate (0.125 M)*	100	47
Indole acetate (0.200 M)*	100	40
Quinone (0.0005 M)	100	97

* Sodium salts.

tors, since examination of the curve for pyruvate oxidation shows a much steeper rise at lower concentrations than does the curve for aerobic utilization of glucose. In the case of a milder inhibitor such as indole butyrate or indole acetate the difference is apparent from the values in Table III. Quinone was equally effective against both the pyruvate and glucose systems.

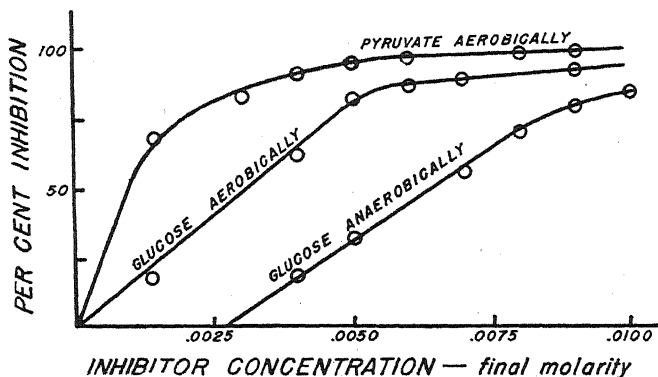


FIG. 2

The Effect of Naphthoxy Heptanoate upon Yeast Metabolism

The aerobic curves represent the inhibition of oxygen uptake of yeast utilizing the substrate indicated.

The anaerobic curve represents the inhibition of carbon dioxide evolution of yeast utilizing the substrate indicated.

Table IV shows that a marked difference exists between the effectiveness of the plant growth substances upon aerobic and anaerobic utilization of glucose by yeast. This difference is emphasized by the curves for aerobic and anaerobic glucose utilization in Fig. 2 where the latter lies well below and has a somewhat smaller slope than the former.

TABLE IV

A Comparison of the Effect of Inhibitors upon Aerobic and Anaerobic Glucose Utilization by Yeast

Compound*	Per Cent Inhibition of Glucose Utilization	
	Aerobic	Anaerobic
1-Naphthoxy valerate (0.007 <i>M</i>)	100	41
2-Naphthoxy heptanoate (0.009 <i>M</i>)	93	56
Indole butyrate (0.090 <i>M</i>)	97	57
Naphthyl acetate (0.045 <i>M</i>)	86	65

* Sodium salts.

In Table V the production of lactic acid by yeast metabolizing glucose under aerobic conditions with and without the addition of inhibitors is summarized. In most cases the production of lactic acid is roughly doubled under the influence of a given inhibitor at a level which gives 100% or nearly 100% inhibition of oxygen uptake. In the case of the two phenylenediamines the rise is six-fold. Quinone, on the other hand, gives a slight inhibition. The effect of concentration of inhibitor is shown for the case of *p*-phenylenediamine in Fig. 3. It will be noted that there is a maximum in the curve after which the produc-

TABLE V

The Effect of Various Inhibitors upon Lactic Acid Production by Yeast

(Values given as $\mu\text{g.}$ of lactic acid produced per flask per hour.

Compound	Average of duplicates.)	
	No Inhibitor	Inhibitor
1-Naphthoxy valerate (0.007 <i>M</i>)*	14.4	38.9
2-Naphthoxy heptanoate (0.009 <i>M</i>)*	16.6	33.8
Indole butyrate (0.090 <i>M</i>)*	14.4	33.1
Quinone (0.0005 <i>M</i>)	17.3	9.0
Naphthoquinone (0.000015 <i>M</i>)	16.9	42.5
<i>p</i> -Phenylenediamine (0.040 <i>M</i>)	12.0	70.0
Dimethyl- <i>p</i> -phenylenediamine (0.040 <i>M</i>)	7.5	40.0

* Sodium salts.

tion begins to fall off. This falling off at higher concentrations of inhibitor is readily demonstrated with the two phenylenediamines, naphthoquinone, quinone, naphthoxy heptanoate, and naphthoxy valerate; although in the last two cases it is not as marked. In the case of indole butyrate, however, the solubility of the compound limits the concentration before such a point is reached.

The values given in Table VI were obtained under the same conditions as those in Table V and show that the production of ethyl alcohol

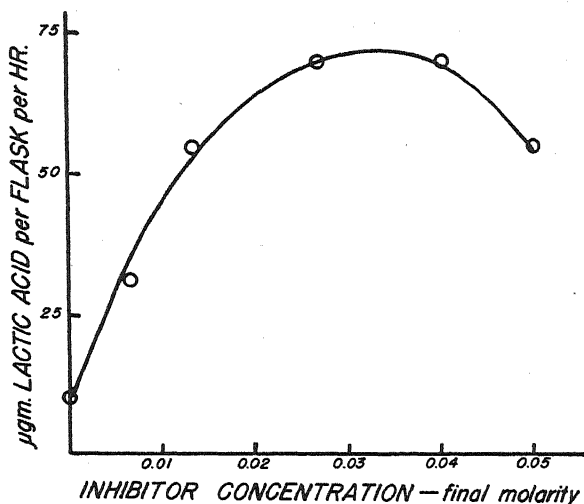


FIG. 3

The Effect of *p*-Phenylenediamine upon Lactic Acid Production by Yeast

is also increased under aerobic conditions by the addition of three of these inhibitors. Again quinone is an exception. The effects of the diamines and naphthoquinone were not studied in this case because the value of the data did not justify the additional complications imposed by the method for the determination of alcohol.

DISCUSSION

The oxidation of pyruvate involves enzymes which lie well along the pathway of carbohydrate metabolism (16) and it marks the division of that metabolism into two parts. That part lying between glucose

and pyruvate can proceed anaerobically, while that portion lying between pyruvate and water and carbon dioxide requires aerobic conditions. Potter (16) has currently reviewed the subject of carbohydrate metabolism and its relation to the cancer problem and in an earlier paper (17) presented a working hypothesis for the investigation of the phenomenon of growth, both neoplastic and normal. A consideration of his arguments and the data cited in their support lead to the conclusion that a substance could produce growth effects by a differential inhibition of the aerobic systems of metabolism. Such an inhibition would result in a depression of the activity of normal cells which gain the major portion of their energy from aerobic systems, and thus allow the immature, non-differentiated cells which can function efficiently under anaerobic conditions, to break through the restraints placed

TABLE VI

*The Effect of Various Inhibitors upon Ethyl Alcohol Production by Yeast**

(Values given as $\mu\text{g.}$ of ethyl alcohol produced per flask per hour.

Compound	Average of duplicates.)	
	No Inhibitor	Inhibitor
1-Naphthoxy valerate (0.007 <i>M</i>)†	414	725
2-Naphthoxy heptanoate (0.009 <i>M</i>)†	436	765
Indole butyrate (0.090 <i>M</i>)†	414	620
Quinone (0.0005 <i>M</i>)	393	331

* Glucose substrate.

† Sodium salts.

upon them by competition with normal cells for structural and energy-yielding materials. A demonstration, therefore, that the enzymes involved in aerobic systems are more sensitive to compounds known to be concerned with carcinogenesis or growth than are the enzymes involved in anaerobic processes furnishes evidence in favor of this point of view.

The data presented in this paper provide such a demonstration. It seems quite clear from the values presented in Tables III and IV and in Fig. 2 that aerobic systems are more sensitive than anaerobic systems.

Pyruvate oxidation should be the most sensitive since a block at this point in the carbohydrate metabolic pathway would be most effective. Aerobic glucose utilization should be the next most sensitive, and when concentrations of inhibitor are used which give complete inhibition of pyruvate oxidation, oxygen uptake on glucose should be

almost completely inhibited, but not entirely, because the transformation of glyceraldehyde to glyceric acid could cause oxygen uptake, and because there is some reason to believe that glucose may be oxidized via the hexosemonophosphate pathway (18). The more effective an inhibitor is, the more difficult it will be to demonstrate this difference, since concentration adjustment must in this case be critical. This fact is well illustrated in the case of quinone where inhibition of both systems occurs at high dilution. Naphthoxy valerate, heptanoate, and propionate are also effective inhibitors, but the curves plotted in Fig. 2 show clearly that by their use differences can be demonstrated between system sensitivities. The rest of the compounds in Table III are relatively mild inhibitors, and differences between the two systems are readily brought out.

When aerobic and anaerobic glucose utilization are compared with respect to sensitivity, results are much more readily obtained and concentration adjustment does not need to be so precise. This may be explained by the fact that the two systems have been more sharply separated than in the situation discussed in the preceding paragraph.

The accumulation of lactic acid as shown in Table V indicates that inhibition is occurring at the pyruvate stage and that pyruvate is acting as an acceptor for the hydrogen from the glyceraldehyde to glyceric acid step. This accumulation is pronounced and consistently present for all the inhibitors tested except for quinone which again may be postulated to be such a good inhibitor that in its presence, at the concentration used, glucose is not even carried as far as lactic acid. In the case of the phenylenediamines the balance of sensitivity of the systems is such that, when proper adjustment of concentration is made, lactic acid accumulates, and as the concentration is increased production of lactic acid begins to fall off, presumably because of inhibition further up the pathway. This effect is also shown by naphthoquinone, naphthoxy heptanoate, and naphthoxy valerate, all effective inhibitors. The effect cannot be demonstrated with indole butyrate, probably because such high concentrations are needed that solubility becomes limiting.

That all of the inhibition does not occur at the pyruvate stage is shown by the fact that ethyl alcohol also accumulates under the same conditions that lead to an accumulation of lactic acid.

The phenomena of low concentrations of plant growth substances stimulating growth and high concentrations inhibiting (19, 20) can

possibly be explained on the basis that, since the aerobic systems are most sensitive, they will be inhibited at low concentrations and growth will result; whereas, at high concentrations the over-all system is inhibited and growth retarded. Since stimulation by a toxic substance at high dilution is a characteristic by no means peculiar to plant growth substances alone, it is necessary to postulate that their *in vivo* action is probably the result not only of the properties demonstrated in this study but also of some other property such as structural configuration, which adjusts the effective concentration of the substance to those levels which afford stimulation until an overwhelming dose is applied.

Caution must be used in transferring the data obtained upon yeast to plant or animal tissue, particularly with regard to concentrations. Yeast is a relatively rugged cell, evolved to survive environmental rigors by itself, whereas, an individual cell within the plant or animal depends upon its associated cells to help it adjust to a change in environment. Hence, it is likely that higher concentrations of a chemical will be necessary to produce a given effect upon yeast than would be necessary in the case of a cell from a multicellular organism.

The above study covers only a small portion of the problem of the control of growth, but it is hoped that it will serve to amplify one aspect of that problem and to stimulate further research into the details of plant metabolism and the action of growth substances thereon.

SUMMARY

The effects of plant growth substances; of *p*-phenylenediamine and dimethyl-*p*-phenylenediamine, which are split-products of the carcinogenic dye *p*-dimethylaminoazobenzene; and of naphthoquinone and quinone upon the metabolism of yeast were investigated. The systems which oxidize pyruvate were found to be most sensitive. Next, in order of sensitivity, were those enzymes concerned in the oxidation of glucose. Least sensitive were the systems involved in the anaerobic utilization of glucose.

These results are interpreted as indicating that a differential inhibition of aerobic systems of metabolism could take place under the influence of these inhibitors and such a possibility is discussed with reference to growth effects.†

† The authors wish to thank Prof. V. R. Potter for valuable advice and criticism.

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A Dietary Factor, Essential for Guinea Pigs
V. Phosphorus and Calcium Content of the Blood and
Muscle During Deficiency¹

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INTRODUCTION

The concentration of calcium ion and inorganic phosphate in the blood are of importance for the maintenance of normal neuromuscular irritability and probably for the deposition of bone salts. Schmitz, *et al.* (1) favored a reciprocal relationship between the calcium and the phosphate in the blood. Their observations favor the view that an increase in phosphate content is accompanied by a simultaneous decrease in the level of calcium. This reduction in the amount of calcium is particularly marked in cases with uremia. Greenwald (2, 3) has criticized this work although he does not deny that there must be mutual influence between the calcium and the phosphate in the blood.

Disturbances in calcium and phosphorus metabolism have been postulated and looked for in many pathological conditions. Abnormal levels may be manifest in rickets, osteomalacia, parathyroid and thyroid dysfunction. The gross effect of vitamin D deficiency is a disturbance of the mineral metabolism. An early symptom is a considerable lowering of the phosphorus in the blood serum and later also a decrease of the calcium level (4). The total serum calcium is not necessarily influenced by a rachitic state, although in severe cases a

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²Based in part on data from a thesis submitted by one of the authors (A. M. F.) as a partial fulfillment of the requirements for the degree of Master of Science. Oregon State College.

decrease (from 9–11 mg. % to about 7–8 mg. %) occurs. Chemical studies of the blood during osteomalacia generally show a low content of serum calcium and inorganic phosphate (5, 6). The early effects of parathyroid hormone action have been investigated (7), leading to the conclusion that the hormone must have a direct solvent action on bone in addition to its effect of increasing the excretion of phosphorus. Aub, *et al.* (8, 9) have demonstrated a greatly increased calcium and phosphorus catabolism in hyperthyroidism. Active untreated thyrotoxicosis in humans results in diminished serum calcium and phosphorus concentrations, which rise after operation (10). The losses of calcium and phosphorus in this condition have been confirmed, but evidence has been presented that they are not due to any direct effect of thyroxin upon bone (11).

Guinea pigs develop a deficiency disease, characterized by degenerative changes in the skeletal musculature when the animals are raised on a diet composed of skimmed milk to which have been added 10% of skimmed milk powder, adequate amounts of copper, iron, carotene, and orange juice (12). The first sign of the deficiency is the development of a stiffness at the wrist joint. A highly active fraction was isolated from raw cream which in a daily dosage of 0.1 μ g. is able to cure the stiffness induced by the skimmed milk diet within five days (13).

As one of the results of the deficiency of this anti-stiffness factor deposits of calcium phosphate appear in many tissues. It was found previously (14, 15) that there is a disturbance in the phosphorus metabolism, during the deficiency, resulting in 1) an abnormal distribution of the acid-soluble phosphorus in the liver and kidneys, and 2) a lowering of the alkaline serum phosphatase. The concentration of the inorganic phosphate in the liver and kidneys in particular was increased far above normal during the deficiency. An increase in this fraction may affect the concentration of the inorganic phosphate in the blood serum and in body tissues other than liver and kidney. The concentration of calcium in the blood and these tissues would also be susceptible to changes under such conditions. The present investigation was undertaken to determine the changes in the phosphorus and calcium concentration in the blood serum and in the muscle of guinea pigs deficient in the anti-stiffness factor.

EXPERIMENTAL

Guinea pigs 11 to 12 weeks of age and weighing from 300 to 350 g. were segregated as to sex and housed in clean cages on autoclaved straw. The animals were fed the diet described by van Wagtendonk (14). This diet is composed as follows:

Skim milk powder	16 g.
Water	84 g.
Ferric chloride	0.25 mg.
Copper sulfate	0.25 mg.
Autoclaved straw	<i>ad lib.</i>
Iodized salt	<i>ad lib.</i>

The diet was given twice a day. To the morning feeding a solution of the water soluble vitamins was added in such a concentration that the average daily intake per animal of the individual vitamins was: Thiamin hydrochloride 0.2 mg.; pyridoxin hydrochloride 0.1 mg.; riboflavin 0.5 mg.; nicotinic acid 1 mg.; pantothenic acid 0.1 mg.; inositol 10 mg.; *p*-aminobenzoic acid 2 mg.; choline 50 mg.; biotin (S.M.A. concentrate S 200) 0.01 mg. To the evening diet was added a solution of the fat soluble vitamins in cotton seed oil. The average daily intake per animal was: β -carotene 150 I.U.; viosterol 40 I.U.; α -tocopherol 0.1 mg.; 2-methyl-1,4-naphthoquinone 0.1 mg. Ascorbic acid (50 mg.) was administered orally once per week.

The animals were sacrificed at various intervals. The blood was obtained by cardiac puncture under nembutal anesthesia. It was sampled in tubes containing heparin (2 mg. per 15 cc. of blood) to prevent clotting. After centrifuging for 10 minutes at 4000 r.p.m. the serum was used for the calcium and phosphorus determination. Calcium was determined according to the method of Sobel and Sklarsky (16). The phosphorus was determined in the supernatant liquid after removal of the calcium oxalate according to the method of Fiske and Subbarow (17) with the modification recommended by Gunther and Greenberg (18).

The rectus femoris was removed and dried at 110°. The muscle was then ashed in a muffle furnace at 600°, the ash taken up in hydrochloric acid and the calcium determined using the method of Sobel and Sklarsky.

The data were analyzed statistically according to the methods of Fisher (19).

RESULTS

Only four age groups of normal animals, 11, 13, 14 and 65 weeks old, were investigated. The results of these analyses are presented in Table I.

From the values obtained with the blood and muscle of deficient guinea pigs (Table II) it can be seen that the inorganic phosphorus in the plasma increased slowly during the deficiency and reached a concentration twice as large as normal in animals which had received the deficient diet for a year. A slight but in most of the cases a sig-

TABLE I

Inorganic Phosphorus and Total Calcium in the Blood and Calcium in the Muscle of Guinea Pigs Raised on a "Stock" Diet

Age in weeks	Weeks on diet	No. of determ.	Inorganic phosphorus mean and S.E. mg. per 100 cc.	Calcium (blood) mean and S.E. mg. per 100 cc.	Calcium (muscle) mean and S.E. mg. per 100 g.
11	8	7	—	9.5 ± 0.2	—
13	10	5	—	9.9 ± 0.6	—
14	11	27	3.0 ± 0.3	10.8 ± 0.3	9.7 ± 1.6
65	62	11	3.3 ± 0.9	9.3 ± 0.5	9.0 ± 1.0

nificant increase in the total calcium concentration of the plasma is found in the later stages of the deficiency.

In the muscle, however, a sharp increase in the calcium content is noted shortly after the guinea pigs receive the deficient diet. Unfortunately, no values were obtained after the animals had received the diet for one week. The significance of the high concentration of calcium found in the muscle after two weeks is therefore doubtful. The higher concentration of the calcium in the muscle was statistically significant in all cases.

The experiments reported in Table III represent a "cure" after administration of varying dosages of the anti-stiffness factor. It was found previously (12) that the low level of the easily hydrolyzable phosphorus fraction in the liver and kidneys of deficient guinea pigs

TABLE II

Inorganic Phosphorus and Total Calcium in the Blood and Calcium in the Muscle of Guinea Pigs Raised on a "Skimmed Milk" Diet

Age in weeks	Weeks on diet	No. of determ.	Inorganic phosphorus mean and S.E. mg. per 100 cc.	Calcium (blood) mean and S.E. mg. per 100 cc.	[Ca] [P] milli- mols per l.	Calcium (muscle) mean and S.E. mg. per 100 g.
14	1	8	4.3 ± 0.2	11.8 ± 0.7	4.6	—
15	2	10	4.3 ± 0.1	10.2 ± 0.6	3.5	28.1 ± 3.3
16	3	16	5.0 ± 0.2	12.9 ± 0.8	5.3	19.9 ± 2.9
24	11	10	4.9 ± 0.2	12.4 ± 0.3	4.9	19.4 ± 2.6
29	16	5	4.7 ± 0.2	12.1 ± 0.7	4.8	16.7 ± 3.2
41	28	11	5.2 ± 0.1	9.3 ± 0.3	3.9	19.4 ± 1.2
70	57	11	6.1 ± 0.2	12.4 ± 0.6	5.9	17.9 ± 1.5

was restored to normal after the administration of small dosages of the anti-stiffness factor. Twenty-nine week old guinea pigs (16 weeks on the deficient diet) were divided into three groups. The first group served as control. The second and third group received the anti-stiffness factor during the last 5 days of the experiment in dosages of 0.01 μ g. (1 unit) and 10 μ g. (1000 units) respectively.³ The concentration of the inorganic phosphorus decreased sharply and even dropped below normal after the administration of 1000 units of the anti-

TABLE III

The Influence of Various Doses of the Anti-Stiffness Factor upon the Inorganic Phosphorus and the Total Calcium in the Blood and the Calcium in the Muscle of Guinea Pigs Raised on a "Skimmed Milk" Diet

Age in weeks	Diet	Weeks on diet	No. of determ.	Inorganic phosphorus mean and S.E. mg. per 100 cc.	Calcium (blood) mean and S.E. mg. per 100 cc.	Calcium (muscle) mean and S.E. mg. per 100 g.
29	skimmed milk	16	5	4.7 \pm 0.2	12.1 \pm 1.3	19.4 \pm 2.6
29	skimmed milk 0.01 γ a.s.f. last 5 days	16	5	3.1 \pm 0.2	12.2 \pm 0.6	17.3 \pm 1.5
29	skimmed milk 10 γ a.s.f. last 5 days	16	10	2.8 \pm 0.1	11.7 \pm 0.6	18.8 \pm 1.2
24	skimmed milk 10 γ a.s.f. every other day during whole experiment	11	11	4.0 \pm 0.2	7.9 \pm 0.6	10.5 \pm 1.0

stiffness factor. No influence of the factor upon either the total calcium concentration in the blood or the calcium content of the muscle could be noted in this short time.

In the last experiment reported in Table III guinea pigs had received the "skimmed milk" diet, supplemented with 1000 units of the anti-stiffness factor every other day during the whole course of the experiment (11 weeks). The inorganic blood phosphorus concentration and the concentration of the calcium in the muscle were normal, while the total calcium content of the blood was significantly below normal.

³ The preparation of this material will be published in a later paper.

DISCUSSION

The influence of the anti-stiffness factor on the concentration of the inorganic phosphorus and the total calcium is of a regulatory nature. As shown in previous investigations (14) its function seems to be that of a controlling mechanism in the phosphorus metabolism. The normal distribution of the acid soluble phosphorus in the liver and kidneys was found to be disturbed when the diet was deficient in the anti-stiffness factor, and resulted in a higher concentration of the inorganic phosphate. The concentration of the inorganic phosphate increases in the blood during the deficiency. Administration of the anti-stiffness factor will reduce the high value to normal levels and the high concentration of the inorganic phosphate is prevented by the administration of the factor with the deficient diet.

The increase in inorganic phosphorus in the blood is accompanied by an increase in the calcium concentration. This simultaneous rise in the concentration of calcium and inorganic phosphate is favorable for the formation of colloidal calcium phosphate. According to McLean and Hinrichs (20) its formation follows when the product $[Ca][P]$ becomes >3 . (The concentrations of the constituents are expressed in millimols per liter.) As can be seen from Table II, this product exceeds the value of 3 a week after the animals were started on the deficient diet. The colloidal calcium phosphate is rapidly removed from the blood (20). A higher concentration and consequent precipitation might follow in the tissues.

Examination of the tissues of guinea pigs deficient in the anti-stiffness factor for a long period of time reveals a gross deposition of calcium phosphate in almost every body tissue (12). Some tissues are heavily calcified. Several determinations were carried out on lung tissue of animals which had received the deficient diet for a year or longer. A mean value of 96.5 mg. calcium per 100 g. tissue (dry weight) was found as compared to 45.6 mg. calcium per 100 g. of lung tissue of normal animals. In one case 406.3 mg. per 100 g. tissue was found.

The inorganic phosphorus and calcium content of the blood returns to normal soon after the administration of the anti-stiffness factor. It is not surprising that the insoluble calcium deposits do not decrease after a short treatment. However, a prolonged administration of large doses (1000 units per day) of the anti-stiffness factor eventually results in a removal of even large deposits from the tissues (personal com-

munication from Dr. Wulzen). In most diseases for which abnormal levels of phosphorus and calcium have been reported the concentrations of these blood constituents are below normal. A deficiency of the anti-stiffness factor has an opposite effect. As yet no insight has been obtained as to the mode of action of the anti-stiffness factor.

SUMMARY

As a result of the deficiency of the anti-stiffness factor the concentrations of the inorganic phosphate and total calcium in the blood are higher than in normal animals or in animals on a deficient diet supplemented with this factor. Colloidal calcium phosphate may then be formed and deposited in the muscle. Administration of the anti-stiffness factor during a short time results in a return to normal values of the high levels of the blood phosphorus and blood calcium. Time is necessary to remove the deposits of calcium phosphate in the tissues.

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Fat Acid Formation During Oil Deposition in Flaxseed ^{1,2}

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INTRODUCTION

Oil is laid down in the flaxseed at a rapid rate soon after flowering. Accompanying oil deposition there is usually a rapid increase in the iodine number of the oil. The results of studies on oil deposition in the maturing flaxseed have recently been summarized (1). It was pointed out that only in the studies of Eyre (2) and Barker (3) was the experimental work carried out in such a way as to follow the true rates of oil deposition and of the accompanying increase in iodine number.

The work described herein is an extension of the type of investigation made by Eyre and Barker. Sufficient seed was collected to follow not only the rate of oil deposition and changes in the iodine number, but also the rate of fat acid formation.

EXPERIMENTAL

In order to obtain oil samples from flaxseed of known age, flax blossoms, which rarely remain open more than a few hours, were tagged with pieces of string. Approximately 50,000 blossoms were tagged from July 2 to July 9 in 1941, and approximately 60,000 from July 6 to July 9 in 1942. Flax bolls were picked at regular intervals, beginning 9 days after flowering in 1941 and 8 days after flowering in 1942. In order to obtain sufficient oil for analysis about 14,000 bolls were picked at the first collection. The number was gradually reduced as oil accumulated in the seed until near ripeness when the number necessary was about 1,500 bolls.

Since oil continues to form, and the iodine number increases when green flax bolls are allowed to air-dry (2, 3), a method of rapid drying which would inhibit these

¹ Presented at the meeting of the American Chemical Society at Cleveland, Ohio, April, 1944.

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changes was necessary. This condition was apparently satisfied at 65°C. in an oven equipped with a circulating fan. The bolls dried in 3 to 4 hours and when the oil was compared with that obtained by other procedures it seemed that no changes in the oil had occurred. The flax bolls collected from tagged blossoms in 1941 were stored in a cabinet maintained at -17°C. until the method of drying was decided upon. The 1942 samples were dried immediately after collection.

The dry flax bolls were hand threshed, the seed weight determined, and the seed ground in a roller mill. The oil content was determined on moisture-free samples from a portion of each meal by extraction with dry ethyl ether. The oil from the remaining meal was extracted with petroleum ether (Skellysolve F). The small oil samples from the oil determination were added to those obtained by petroleum ether extraction and the solvent was removed by warming *in vacuo*. Analysis of the oils was carried out by methods previously described (4). The thiocyanogen number of each of the 1941 oil samples was determined with two thiocyanogen solutions, and the 1942 samples with three such solutions. Two separate determinations of the iodine number were also made. Free fat acids in the oils were determined by titration in alcohol prior to saponification.

Bison was the variety grown in 1941. B. Golden, a variety which produces an oil with an iodine number approximately 10 points higher than Bison, was grown in 1942.

DISCUSSION OF RESULTS

The analytical results obtained on the samples collected each year are shown in Tables I and II.

TABLE I

Analytical Results on Seed and Oil from Flax at Different Stages of Growth (1941)

Growing period. Tagging to collection	Weight per 1000 seeds*	Oil content of seed*	Oil per 1000 seeds	Proportion of total oil accumulated	Free fat acids†	Un-saponifiable	Saturated acids	Iodine number	Thio-cyanogen number
days	g.	per cent	g.	per cent	per cent	per cent	per cent		
9	1.73	10.5	.182	8.1	2.76	2.34	13.3	150.1	100.4
12	2.52	24.6	.620	27.5	2.33	1.34	13.4	163.8	108.1
14	2.82	26.4	.745	33.0	2.19	1.36	13.3	161.8	105.5
17	3.72	37.9	1.408	62.6	1.14	1.15	12.7	157.0	104.0
19	4.38	39.2	1.716	76.2	0.45	1.01	11.8	155.2	103.0
21	5.17	40.5	2.093	92.9	0.51	1.03	11.2	156.3	104.4
24	5.55	40.6	2.253	100.0	0.42	1.02	10.7	155.0	103.6
27	5.40	40.3	2.173	96.6	0.40	.99	10.6	156.8	104.8
32	5.59	40.1	2.244	99.5	0.45	.97	10.3	155.7	103.2

* Moisture-free basis.

† Calculated as stearic acid.

TABLE II

Analytical Results on Seed and Oil from Flax at Different Stages of Growth (1942)

Growing period. Tagging to collection	Weight per 1000 seeds*	Oil content of seed*	Oil per 1000 seeds	Proportion of total oil accumulated	Free fat acids†	Un-saponifiable	Saturated acids	Iodine number	Thio-cyanogen number
days	g.	per cent	g.	per cent	per cent	per cent	per cent		
8	1.56	9.3	.145	4.5	2.75	2.48	15.4	135.4	93.1
10	2.20	16.3	.359	11.2	1.73	2.01	15.4	141.0	95.5
12	2.57	25.0	.643	20.0	1.55	1.31	13.3	158.2	104.3
14	3.05	33.2	1.013	31.5	1.00	1.15	13.4	173.5	110.7
16	3.72	38.6	1.436	44.7	.72	1.08	11.3	182.3	115.5
18	4.34	42.5	1.845	57.4	.68	.88	10.7	187.9	119.9
20	4.85	44.5	2.158	67.1	.65	.97	10.0	192.7	122.1
22	5.40	45.9	2.479	77.1	.72	1.04	9.9	193.4	123.5
24	5.77	45.8	2.643	82.2	.90	1.06	9.1	195.2	125.4
27	6.52	46.7	3.045	94.7	.48	.81	9.1	198.1	126.9
30	6.96	46.2	3.216	100.0	.40	.90	8.9	197.3	126.4
33	7.00	44.6	3.122	97.1	.45	1.00	8.4	195.2	125.8
37	7.10	44.5	3.160	98.3	.38	.96	8.8	194.6	125.2
40	7.26	44.0	3.194	99.3	—	—	—	193.3	—

* Moisture-free basis.

† Calculated as stearic acid.

Free fat acids were highest in the oils from green flaxseed and decreased in percentage as seeds matured. Eyre (2) and Barker (3) observed similar changes. Lipase activity (5) of flaxseed meal has been found to decrease as the seeds approached maturity. If we assume that the action of lipase is primarily that of esterification in oil formation, these results may be interpreted to support the concept of Ivanov and Klokov (6) that free fat acids are first formed and these in turn are esterified with glycerol.

The percentage of unsaponifiable matter decreases, as also shown by Theis, Long, and Beal (7), when flaxseed matures. Plant pigments appear to be high in immature flaxseed. The first samples picked each year showed a faint greenish tinge. The intensity of the green color increased until near the twentieth day after flowering then diminished until the seed was ripe, when the seeds took on their normal color. No doubt chlorophyll was present in the unsaponifiable portion measured, but much of the green pigment remained in the soap solution when the unsaponifiable was extracted.

Oil deposited (Fig. 1) at a more rapid rate than reported heretofore, and oil continued to be formed after maximum percentage of oil in the seed had been reached. Maximum oil (weight of oil per 1000 seeds) was found before the last collection of seed each year, but the values at 24 days in 1941 and at 30 days in 1942 (Tables I and II, Fig. 1) appear to be out of line. This is likely due to an inaccurate determination of the seed weight. It must also be recognized that plots are not

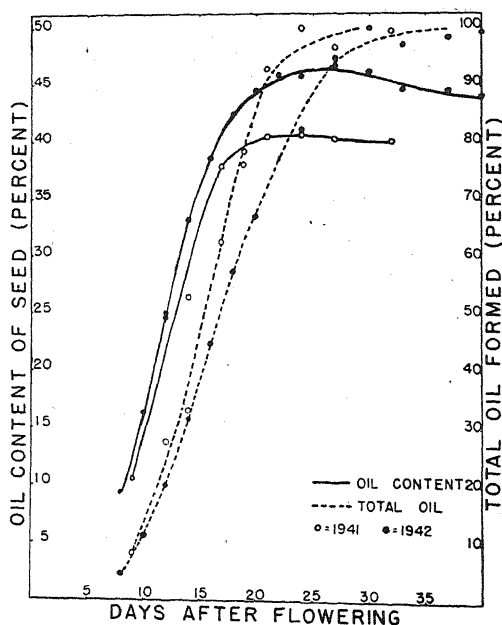


FIG. 1

Rate of Oil Deposition as Shown by Percentage of Oil in the Seed and Proportion of Total Oil Formed

perfectly uniform, and the blossoms were not all tagged the same day, so not all samples developed under identical conditions. All the 1942 samples were tagged within $3\frac{1}{2}$ days and growing conditions were quite uniform, but in 1941 the samples were tagged over 8 days and wide variations in temperature occurred during the oil forming period. For this reason the 1942 data are considered more reliable than the 1941 data.

Changes in the iodine number of the oils during deposition (Fig. 2) differed markedly in the two years. The rate of increase in 1942 was much more rapid than found by Eyre (2), Barker (3), or by McGregor, Lehberg, and Geddes (8). These results, as well as those on oil deposition rates, are influenced by weather conditions and have been discussed (9) in connection with flaxseed production.

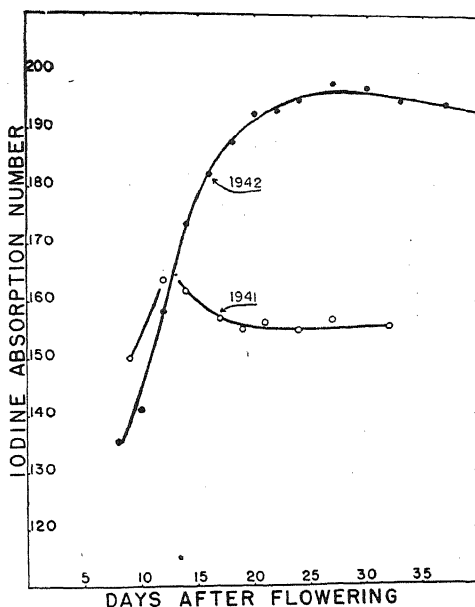


FIG. 2

Changes in the Iodine Absorption Number of Oil from Flaxseed during Oil Deposition

The fat acid composition of the oils obtained in each of the two years is shown in Tables III and IV. Fat acid composition is reported in terms of glycerides, but, to simplify the discussion, reference to these will be made by name of the individual fat acids. The 1942 results, where marked changes in the percentage composition of the fat acids accompanied the increase of the iodine number, are plotted in Figure 3.

Since composition of linseed oil is related to the iodine number (10), large variations in composition would not be expected in 1941 when the range in iodine number was only 13.7 points. The increase in iodine

number which occurred between the 9th and 12th day was accompanied by a percentage increase in linolenic acid and a decrease in oleic acid (Table III). At 14 days when the iodine number was lower, the percentage of oleic acid increased and that of linolenic acid decreased. During the latter part of the oil forming period, when the iodine number remained nearly constant, variations in the unsaturated acids were small. The percentage of saturated acids, however, decreased significantly as oil formed in the seed.

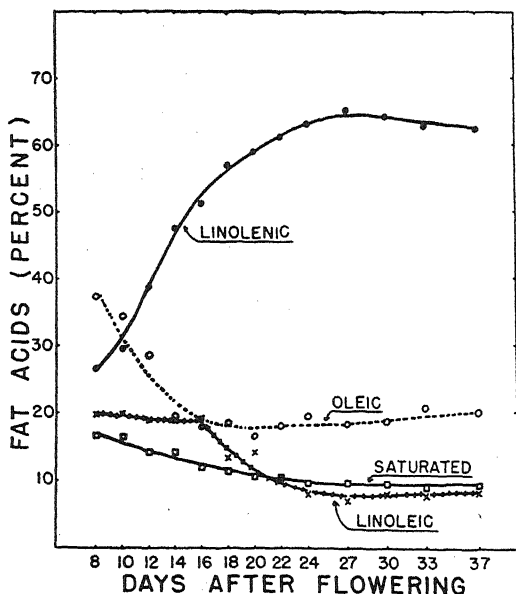


FIG. 3

Changes in the Relative Amounts of Fat Acids in Oils of Flaxseed during the Period of Oil Deposition

In 1942 the percentage of the fat acids (Fig. 3) changed markedly during the oil formation period. Linolenic acid increased steadily from 26.5% at 8 days to 65.2% at 27 days after flowering, when the maximum iodine number was reached, then it decreased slightly. Oleic acid dropped sharply from 37.3% at 8 days after flowering to 19.5% at 14 days, then remained fairly constant. Linoleic acid remained nearly constant until 16 days, then decreased until about 24 days,

TABLE III

Changes in Fat Acid Composition during Oil Formation Period (1941)

Growing period. Tagging to collection days	Saturated per cent	Composition of Glycerides		Linolenic per cent
		Oleic per cent	Linoleic per cent	
9	14.3	32.2	19.8	33.7
12	14.2	27.7	13.6	44.5
14	14.1	25.9	19.7	40.3
17	13.5	29.9	18.9	37.7
19	12.4	31.2	21.6	34.8
21	11.8	32.6	19.4	36.2
24	11.3	33.2	21.1	34.4
27	11.2	33.0	19.8	36.0
32	10.8	32.0	24.3	32.9

TABLE IV

Changes in Fat Acid Composition during Oil Formation Period (1942)

Growing period. Tagging to collection days	Saturated per cent	Composition of Glycerides		Linolenic per cent
		Oleic per cent	Linoleic per cent	
8	16.5	37.3	19.7	26.5
10	16.4	34.3	19.8	29.5
12	14.0	28.5	18.8	38.7
14	14.2	19.5	18.8	47.5
16	11.9	17.8	19.1	51.2
18	11.3	18.5	13.2	57.0
20	10.5	16.5	14.0	59.0
22	10.5	18.0	10.2	61.3
24	9.6	19.5	7.8	63.1
27	9.6	18.3	6.9	65.2
30	9.4	18.6	7.8	64.2
33	8.9	20.7	7.6	62.8
37	9.3	20.1	8.2	62.4

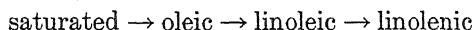
when it leveled off. The saturated acid content was somewhat erratic in the first four samples, but there was a gradual decrease.

These results (1942) show far greater changes in the percentage of the fat acids of linseed oil during the period of oil formation than those of Theis, Long, and Beal (7). The composition of the oils is also quite different, but this is expected because there has been notable improvement in the thiocyanometric technique and in the determination of the saturated acids since they reported their work. Theis, Long, and Beal

found linoleic acid to increase, whereas Ivanov and Klovov (6) found it to decrease. Both groups report a decrease in the percentage of saturated acids in oil as the flaxseed approaches maturity, but Morrell (11) found the saturated acids to increase.

Since Eyre (2) and Barker (3) found the iodine number to increase for several days after the maximum percentage of oil in the seed had been reached, they concluded that the increase in iodine number and oil formation are independent processes. This may well be true, but they failed to recognize that oil may continue to be formed as long as seed weight increases (see Fig. 1) even though the percentage of oil had reached a maximum. From their own data it is likely that oil continued to form throughout the period of increase of the iodine number. The results in Figures 1 and 2 show that the iodine number did not continue to increase after the total oil had been deposited in the seed. On the other hand, there is conclusive evidence that the iodine number may decrease (Figs. 1 and 2, and Ref. 1, 12) before all of the oil has formed.

The increase in the iodine number of linseed oil during the period of oil formation, and the changes in percentage of the fat acids (6, 7) has led some writers to describe the change as a desaturation process. This implies a dehydrogenation of fat acids already laid down in the seed, and several workers (2, 3, 6, 7, 12) have interpreted their results as evidence that the first acids formed are saturated. An actual conversion of one fat acid into another by a stepwise dehydrogenation, *i.e.*



seems a plausible explanation for the results cited and those in Fig. 3. Such a mechanism could account for the changes shown in Table III if we assume that the process is reversible.

The argument cited is based on observations which consider relative changes in the fatty acids, but it fails to take into account the weight increase in oil in successive collections of seed up to maximum oil deposition. When the fat acids are calculated to total amounts (as weight per 1000 seeds) formed during the period of oil deposition (Tables V and VI, Fig. 4) quite a different picture is presented. In Table V (1941 results) it is shown that all of the different fat acids increased in total amount. The total fat acids formed in the 1942 growing season, when all of the plants developed under more nearly similar conditions (Fig. 4), reveal that linolenic acid was formed at

TABLE V

Rate of Fat Acid Formation (1941)

Growing period. Tagging to collection days	Total glyc- erides per 1000 seeds g.	Glycerides per 1000 seeds			
		Saturated g.	Oleic g.	Linoleic g.	Linolenic g.
9	0.177	0.025	0.057	0.035	0.060
12	0.612	0.087	0.170	0.083	0.272
14	0.735	0.104	0.190	0.145	0.296
17	1.392	0.188	0.416	0.263	0.525
19	1.699	0.211	0.530	0.367	0.591
21	2.072	0.244	0.676	0.402	0.750
24	2.230	0.252	0.740	0.471	0.767
27	2.155	0.241	0.711	0.427	0.776
32	2.220	0.240	0.710	0.540	0.730

TABLE VI

Rate of Fat Acid Formation (1942)

Growing period. Tagging to collection days	Total glyc- erides per 1000 seeds g.	Glycerides per 1000 seeds			
		Saturated g.	Oleic g.	Linoleic g.	Linolenic g.
8	0.142	0.024	0.053	0.027	0.038
10	0.351	0.058	0.120	0.069	0.104
12	0.634	0.100	0.181	0.085	0.268
14	1.001	0.142	0.195	0.188	0.476
16	1.420	0.170	0.242	0.290	0.718
18	1.828	0.206	0.313	0.293	1.016
20	2.137	0.225	0.382	0.241	1.289
22	2.453	0.258	0.451	0.235	1.509
24	2.614	0.252	0.510	0.205	1.647
27	3.020	0.290	0.564	0.190	1.976
30	3.187	0.298	0.601	0.243	2.045
33	3.091	0.275	0.640	0.235	1.941
37	3.128	0.291	0.629	0.256	1.952

an astonishingly rapid rate until the maximum iodine number was reached. Whereas the percentage of oleic and saturated acids decreased as oil formed (Fig. 3), the total amounts of these acids actually increased. In the case of linoleic acid there was a small decrease in the total amount in 1942 and it came during the growth period when oil was being rapidly formed and when there was a rapid increase in the iodine number. Whether or not the decrease is real is open to question. The actual decrease was from almost 0.3 g. per 1000 seeds to near 0.2 g. (Table VI). This is more than should result due to errors of precision

in the method of analysis, although the probable error in the determination of linoleic acid is almost twice that of linolenic or oleic acids. The linoleic acid content fell to an unusually low level for linseed oil. If the decrease in the weight of linoleic acid is real it is strong evidence of a conversion of one fat acid to another; in this case

linoleic \rightarrow linolenic.

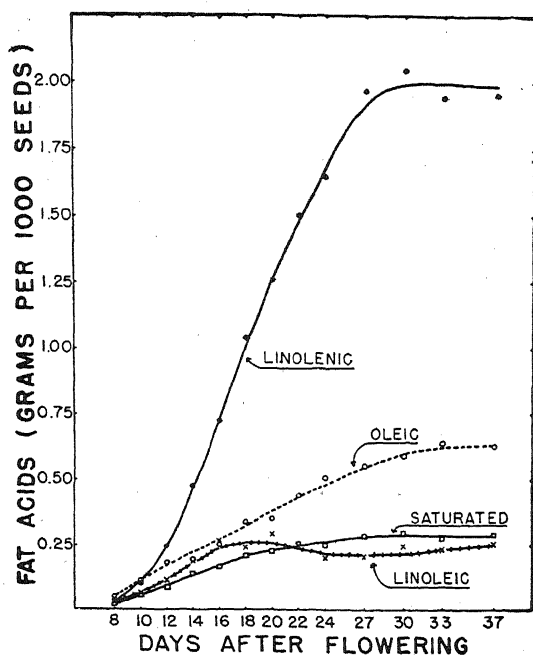


FIG. 4

Rate of Formation of Fat Acids during the Period of Oil Deposition

A dehydrogenation mechanism for the formation of unsaturated fat acids in oleaginous seeds may appear the most likely. Schoenheimer and Rittenberg (13) have given conclusive evidence that the animal can desaturate fat acids (although no linoleic acid was formed), and it may seem probable that the plant can do likewise. Aside from the observed decrease in linoleic acid in 1942, which is strong evidence for dehydrogenation of this acid, a mechanism of formation of fat acids,

which appears just as likely, is to assume that each of the fat acids are preformed and that the rate is modified throughout the period of oil accumulation. The results do not, in the opinion of the author, indicate that only saturated acids are those first formed and the unsaturated acids arise by a stepwise dehydrogenation. If saturated acids are those first laid down, the iodine number of the oil first deposited should be zero. After 8 days of growth less than 5% of the total oil had been deposited (Fig. 1), yet the iodine number was above 135. The figures showing the rate of increase of the iodine number during each season (Fig. 2), as well as those showing the rate of formation of the fat acids (Fig. 4), do not indicate that the oil first formed is composed of saturated glycerides. Furthermore, if dehydrogenation of saturated acids occurs, it is specific for C_{18} acids. There are saturated acids in linseed oil other than 18 carbon acids, but all of the known unsaturated acids are C_{18} .

SUMMARY AND CONCLUSIONS

Flax bolls were tagged at the time of flowering and samples collected at regular intervals during the period of oil formation in each of the 1941 and 1942 seasons. Sufficient material was obtained so that the rates of oil formation, and changes in iodine number and fat acid composition could be followed throughout the period of oil deposition.

Oil deposition was rapid each season and oil continued to form for about 5 days after the maximum percentage of oil in the seed had been reached.

In 1942 the iodine number increased rapidly from 135.4 eight days after flowering to a maximum of 198.1 twenty-seven days after flowering. In 1941 the iodine number increased during the early stages of growth then dropped sharply long before all of the oil accumulated in the seed.

Free fat acids and unsaponifiable matter decrease in percentage as the oil accumulates. Changes in the percentage of the fat acids in the oils from the 1941 samples were small and paralleled changes in the iodine number. In 1942 linolenic acid increased in percentage until the maximum iodine number was reached. The percentage of oleic acid dropped sharply in the early part of the growing period, then leveled off to a near constant value. Linoleic acid remained nearly constant until the 14th day of growth, then decreased in percentage until about

the 24th day. The percentage of saturated acids decreased throughout the period of oil formation in both 1941 and 1942.

When the fat acids are calculated to show the actual amount formed (as grams per 1000 seeds), the picture is quite different from that showing changes in per cent. *Each fat acid, except for a slight decrease at one point in the amount of linoleic acid, increased in total amount throughout the period of oil formation.*

The results presented may be interpreted to indicate that unsaturated fat acids are formed by desaturation, but it appears doubtful whether this is the only mechanism. It seems equally probable that unsaturated acids are preformed.

Acknowledgment

The author is indebted to Mr. T. E. Stoa, Agronomist, for providing the flax plots, to Mr. L. L. Nesbitt of this laboratory for laboratory analyses, and to numerous students who assisted in tagging flax blossoms. Mr. Arnold Stoutland and Mr. Brice Nesbitt assisted in collection of the samples and threshing the seed.

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Iron in Anemic Rat Tissues¹

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INTRODUCTION

Data on the iron content of tissues of rats with nutritional anemia have been published by Cunningham (2), Josephs (4), Elvehjem and Sherman (3), Wakeham and Halenz (12), and Austoni, Rabinovitch, and Greenberg (1). Using animals made copper or iron deficient as described by Schultze (8), and with the aid of iron methods developed in this laboratory, these results have been augmented, and certain conclusions regarding iron metabolism are presented here.

EXPERIMENTAL

Experimental Animals

Animals used in these studies were of four types; normal, iron deficient, iron deficient receiving iron therapy, and copper deficient. All animals received milk as their sole source of food from birth and were kept after weaning in glass cages in most cases. They were given 30 ml. of milk per day plus the following daily supplements:

Normal rats: 0.1 mg. Cu^{++} + 0.05 mg. Mn^{++} + 0.5 mg. Fe^{+++}

Iron deficient rats: 0.1 mg. Cu^{++} + 0.05 mg. Mn^{++}

Copper deficient rats: 0.05 mg. Mn^{++} + 0.5 mg. Fe^{+++}

Iron therapy rats: 0.1 mg. Cu^{++} + 0.05 mg. Mn^{++} till deficient; then 0.5 mg. Fe^{+++} in addition for five days before analysis.

The characteristics of these groups of rats are shown in Table I. The iron deficient group differs only in total iron content of blood from the normals, but the copper deficient group weighs less and has very much enlarged livers. Enlarged spleens can occur in any group but are much more common in iron therapy and copper

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TABLE I
General Characteristics of Experimental Animals

	Normal rats	Fe deficient rats	Fe therapy rats	Cu deficient rats
Number of animals	20	24	18	10
Age (days)	88.5 \pm 5.1	74.3 \pm 3.7	74.7 \pm 3.6	67.7 \pm 2.5
Weight (g.)	101.6 \pm 3.4	93.5 \pm 4.0	93.8 \pm 4.7	69.7 \pm 3.7
Total blood Fe (mg./100 ml.)	49.8 \pm 1.2	14.6 \pm 0.8	35.4 \pm 1.1	18.6 \pm 1.7
Hematocrit (%)	44.6 \pm 1.3	18.9 \pm 0.6	38.0 \pm 2.7	19.4 \pm 1.9
Total cell Fe (mg./100 ml.)	109.1 \pm 1.7	74.4 \pm 3.3	87.3 \pm 3.6	99.4 \pm 2.2
Liver weight (g.)	3.57 \pm 0.15	3.35 \pm 0.13	3.34 \pm 0.08	5.49 \pm 0.20
Spleen weight (g.)	0.325 \pm 0.046	0.352 \pm 0.042	0.443 \pm 0.037	0.488 \pm 0.054

deficient rats. In agreement with Smith and Medicott (10), red cells in copper deficiency are much less hypochromic than those in iron deficiency. No differences in the two sexes in any group were noted that could not be related to size, except that females in the iron deficient group were significantly more anemic. Significant correlation in the iron therapy group indicated that older (and heavier) animals recover less quickly from anemia.

It should be noted that a copper deficiency anemia in the presence of adequate iron is difficult to realize. Repeated attempts to obtain anemia with milk containing less than 0.1 mg./kg. Cu have been unsuccessful. If, however, the milk is treated with H₂S, see Schultze (8), anemia results. This may be due to non-availability of copper sulfide or to some other effect of H₂S. The anemia, unlike that in iron deficiency, is often quite moderate, and some animals die before they become anemic at all.

Preparation of Tissues. No iron was fed to the animals for 24 hours before analysis. They were anesthetized and blood obtained by heart puncture. The liver and spleen,

TABLE II
Total Iron Content of Rat Tissues

Tissue:	Normal rats	Fe deficient rats	Fe therapy rats	Cu deficient rats
Serum (γ /100 ml.)	362 \pm 27	141 \pm 11	234 \pm 20	160 \pm 25
Liver (mg./100 g.)	22.2 \pm 1.8	2.6 \pm 0.1	5.9 \pm 1.0	14.6 \pm 1.6
Spleen (mg./100 g.)	40.9 \pm 5.9	12.1 \pm 1.2	18.7 \pm 1.3	24.7 \pm 4.6
Muscle (mg./100 g.)	2.11 \pm 0.16	1.17 \pm 0.15	1.68 \pm 0.14	1.71 \pm 0.10
Bone marrow (γ per animal)	19.4 \pm 1.4	7.5 \pm 0.6	19.3 \pm 1.5	9.5 \pm 0.8

bone marrow as obtained from the six long bones (femora, tibiae, humeri), and a weighed portion of leg muscle were dispersed in water with an all glass grinder and made up to volume. Aliquots were taken for analysis by methods described elsewhere (9). The results of the analyses for total iron are shown in Table II; those for free iron in Table III. The percentage of total iron measured as free iron is shown in Table IV. All data are presented on a moist tissue basis.

TABLE III
Free Iron Content of Rat Tissues

Tissue:	Normal rats	Fe deficient rats	Fe therapy rats	Cu deficient rats
Serum (γ /100 ml.)	236 ± 19	81 ± 7	157 ± 15	74 ± 9
Liver (mg./100 g.)	2.67 ± 0.23	0.80 ± 0.04	1.09 ± 0.10	1.80 ± 0.12
Spleen (mg./100 g.)	3.54 ± 0.58	1.22 ± 0.09	1.42 ± 0.11	1.81 ± 0.16
Muscle (mg./100 g.)	0.92 ± 0.09	0.52 ± 0.05	0.66 ± 0.06	0.50 ± 0.03
Bone marrow (γ per animal)	1.79 ± 0.17	0.91 ± 0.05	1.85 ± 0.16	0.99 ± 0.06

TABLE IV
Percentage of Total Iron Measured as Free Iron

Tissue:	Normal rats	Fe deficient rats	Fe therapy rats	Cu therapy rats
Serum	63.5 ± 8.5	62.9 ± 6.9	61.8 ± 1.1	51.8 ± 5.3
Red cells	13.9 ± 0.9	14.6 ± 1.6	12.1 ± 1.0	11.4 ± 1.0
Liver	12.4 ± 0.5	31.7 ± 2.3	20.7 ± 1.6	13.0 ± 0.8
Spleen	9.0 ± 1.2	11.2 ± 1.3	7.6 ± 0.6	8.1 ± 0.6
Muscle	45.1 ± 4.3	48.8 ± 6.1	41.0 ± 3.9	30.0 ± 2.3
Bone marrow	9.8 ± 1.0	14.0 ± 1.5	9.9 ± 0.9	10.7 ± 1.0

Solubility and Dialyzability of Iron in Tissues. Tissue extracts from normal rats prepared as above were electro dialyzed in a small lucite cell until the conductivity was constant. The tissue was then redispersed and total and free iron measured. The solubility of iron in these tissues was determined in water and in 0.2 M HCl. The iron which did not precipitate on centrifuging at 5000 r.p.m. during one-half hour was considered soluble. The results are presented in Table V.

Calculation of Iron Fractions. For present purposes, iron of tissues was considered to consist of three types—blood iron, present because of blood in the tissue, and dependent in amount on blood iron level

TABLE V

Dialyzability and Solubility of Iron in Normal Rat Tissues

	% free before Electro- dialysis	% free after Electro- dialysis	In water		In 0.2 M HCl	
			% of total Fe soluble	% of free Fe soluble	% of total Fe soluble	% of free Fe soluble
Red cells	9.4	9.1	93	88	69	87
Liver	12.1	19.5	100	93	94	100
Spleen	7.8	5.8	62	59	48	100
Muscle	42.5	37.5	57	38	63	47

and weight of the tissue; stored iron, which could be utilized for hemoglobin synthesis; and tissue iron, which was independent of blood iron level and iron storage and not available for hemoglobin synthesis. Iron deficient and iron therapy rat tissues were believed to have no stored iron, so from a comparison of their iron contents, the amount of blood iron present and the tissue iron could be calculated. Normal and copper deficient rat tissues contained in addition stored iron and this too could be calculated by difference. It is recognized that these three fractions are not necessarily distinct nor invariable, nor are they chemical and physiological entities, but the figures calculated below represent the best possible estimate for practical purposes of three functionally different types of iron in tissue. Similar calculations were applied to the free iron levels to determine the approximate percentage of tissue and stored iron that will react with thiocyanate.

DISCUSSION

Serum Iron. The serum iron is 50 to 65% reactive with thiocyanate; consequently a large portion of it cannot be derived directly from hemolyzed cells which react only 12 to 15% with thiocyanate. If the apparent serum iron is corrected for the maximum possible amount of iron from hemolyzed cells, the average minimum true serum iron levels are still 216, 65, 146, and 63 γ /100 ml. in the normal, iron deficient, iron therapy, and copper deficient rats, respectively. The serum iron is almost directly proportional to total blood iron and shows significant correlation with free spleen iron. In these four groups of animals, it may be concluded tentatively that the rate of hemolysis *in vivo* is more significant than any other single factor in determining the serum iron level. (In one copper therapy animal analyzed, the serum iron

level was extremely high, so this explanation does not hold in that case.) From the high proportion of serum iron that is free, it must be concluded that serum iron is either ferrous ion or complex or a ferric complex. Ferric ions can not exist in measurable amounts in tissues because of the low solubility product of ferric hydroxide.

Cell Iron. The percentage of cell iron that reacts with thiocyanate varies somewhat with hemoglobin level, so that the free iron level is more nearly constant than the hemoglobin iron level per unit volume of cells. Although the purest rat hemoglobin crystals we have obtained still contained 6% free iron, a fraction left after crystallization contained 30% of the total as free iron. Little of the free iron is electro-dialyzable but its acid solubility is independent of the formation of insoluble hematin from hemoglobin. Considering these facts, it is probable that hemoglobin itself does not react with thiocyanate. The free cell iron fraction is apparently the "easily split" iron studied by Moore, *et al.* (7). The amount of iron measured by us is higher than that found by Moore, but it is noted that his figures depended on the method of analysis.

Bone Marrow Iron. The bone marrow sample here measured may be assumed to represent 50% of the tissue active in hematopoiesis. Significant negative correlations of bone marrow iron with serum iron, spleen free iron, and muscle iron were found in normal rats. Bone marrow was calculated to contain about 12 ml. blood per 100 g. tissue. About 5 γ of the iron in bone marrow samples taken is not affected by blood iron level and is considered tissue iron; about 3.5 γ of iron can be stored in bone marrow. There is an excess of about 10 γ iron in iron-therapy bone marrow which is present because of increased hematopoiesis. From the total blood iron increase during five days therapy (about 1.5 mg. per rat), the average time for formation of a red cell is estimated at 40 to 100 minutes.² The tissue and excess iron in bone marrow react about 8% with thiocyanate; the stored iron about 3%.

Liver Iron. Only about 50 γ of iron in rat liver is independent of state of iron metabolism, but about 600 γ can be stored. Liver contains 8 ml. blood per 100 g. tissue. Three out of twenty iron therapy

² The excess iron in all bone marrow in the rat was estimated at not less than 7.5 γ nor more than 20 γ . The excess must have been converted into hemoglobin between 200 and 75 times during 5 days, and the time of conversion must have been 36 to 96 minutes. The time of conversion of iron is equal to the time of formation of a red cell if the iron content of red cells is uniform.

rats showed evidence of iron storage in liver; it is therefore concluded that the rate of absorption of iron under these conditions is approximately equal to the maximum possible rate of hematopoiesis during iron therapy. Stored iron of liver reacts about 9% with thiocyanate and is independent of liver weight. Tissue iron of liver reacts about 40% with thiocyanate. The free iron of normal liver extracts increased with time, presumably because of some autolytic process, see McFarlane (5). It is almost completely soluble in water.

Spleen Iron. About 55 γ of iron is stored in rat spleen. Because only about 5% of this reacts with thiocyanate, ferritin, which reacts 23% with thiocyanate (9), does not appear to be of importance in storage of iron in rats. Iron storage in spleen is closely correlated with that in liver, and both react with thiocyanate to the same extent as does FeOOH (9). Spleen contains 30 to 35 ml. of blood per 100 g. tissue, and about 25 γ of tissue iron. The latter reacts about 3% with thiocyanate. The free iron of spleen shows more significant correlations with iron content of other tissues than does any other iron level measured.

Muscle Iron. Iron levels of muscle are directly proportional to blood level in normal, iron deficient and iron therapy rats. Thus no iron is normally stored in muscle. In copper deficient rats, a small amount of storage (0.2 mg./100 g. muscle) is indicated by a high iron content and low reactivity with thiocyanate. Muscle contains 2 ml. blood per 100 g. tissue and tissue iron to the extent of 0.9 mg. per 100 g. The latter is about 55% reactive with thiocyanate and because of the relative insolubility of free iron of muscle is presumed to be an iron-protein complex.

Copper deficiency has previously been shown to have little effect on iron absorption (3). It has no effect on iron storage except that an abnormal amount of stored iron may appear in muscle. That the effect of lack of copper in producing anemia is not due to non-availability of iron is shown by the only moderately hypochromic character of red cells in copper deficiency. Copper probably does not labilize stored iron, since remission from anemia of at least one other type also causes high serum iron values (6). Stein and Lewis (11) and Smith and Medlicott (10) have shown that copper has an erythropoietic function independent of hemoglobin formation. Since remission from anemia of any type must necessarily involve utilization of iron in hemoglobin synthesis, this erythropoietic function might well be the only way in

which copper affects recovery from anemia were it not that the red cells in copper deficiency are abnormal. The early mortality not associated with anemia, the lack of growth, and the enlarged liver and spleen of copper deficient rats indicate that lack of copper in the diet has a general debilitating effect. The inability to regenerate hemoglobin at the normal rate is only one of the evidences of this general ill health.

SUMMARY

1. The free and total iron levels of tissues from rats with nutritional anemia have been measured, and amounts of blood iron, stored iron, and tissue iron calculated.

2. Stored iron in all tissues analyzed from its reaction with thiocyanate appeared to be FeOOH .

3. The time of formation of the red cell is 40 to 100 minutes as calculated from iron turnover in bone marrow.

4. The anemia of copper deficiency is only one of several unfavorable effects of lack of this mineral. No evidence was obtained of a specific role of copper in iron metabolism.

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Simple Apparatus for the Study of Blood-Gas Equilibria *

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INTRODUCTION

The methods of blood-gas equilibration commonly used today (1, 2, 3), employ tonometers or saturators (4) of Barcroft's design (5, 6, 7). More recently Barcroft (8) designed a compact tonometer which was modified by Irving and Black (9). Several types of tonometers have been employed in spectrophotometric studies (10, 11, 12, 13, 14, 15). A number of other tonometers have been described (4, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25). Several (16, 26, 27, 28) were designed to measure the gas tension present in the liquid entering the apparatus.

The methods usually employed involve complex, cumbersome, and expensive equipment. The technic and apparatus described here are simple and convenient.

APPARATUS

Syringes of 50 or preferably 100 ml. capacity are used as tonometers. For convenience in transferring the gas phase, syringes with eccentric tips are desirable. The tip of the syringe is connected by heavy rubber tubing to a glass needle adapter made from the end of a small tuberculin syringe. The adapter is fitted with a No. 23 needle which is plugged with a rubber stopper (Fig. 1).

Syringes of 5 or 10 ml. capacity fitted with needles are used for transferring and adding small measured amounts of gases to the

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syringe tonometer. Aspirator bottles may be used for storing gases or gas mixtures, or the gases may be obtained directly from rubber tubing attached to the tank valves.

Gas bags of 0.0033 inch thick Koroseal² can be easily made and have been found convenient for storing gas mixtures. Diffusion of gases through this material is slow.

Gas sample holders filled with slightly acidic concentrated sodium sulfate solution are used for storing the equilibrated gas phases (Fig. 1).

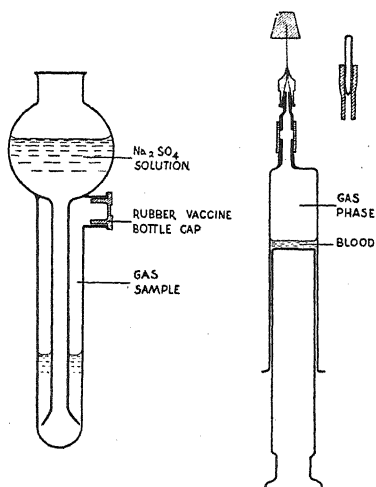


FIG. 1

Gas Sample Holder and Syringe with Adapters for Use as a Tonometer

They are made from Pyrex 100 ml. extraction flasks sealed to large Pyrex test tubes. The inner tube is flared to assist in removal of residual gas by inversion of the vessel.

Haldane gas analysis apparatus or *Scholander quick analyzers* are used for analyzing the equilibrated gas phases. In order to avoid considerable exchange of gases between the liquid and gas phases in the quick analyzers, the volume of the liquid which may come in contact with the gas is reduced to a minimum. The analyzer for absorption of oxygen and carbon dioxide is made from a 10 ml. Mohr pipette with

² We are indebted to C. D. Segner, Plastic Materials Division of the B. F. Goodrich Company, who kindly supplied this material.

jacket and a 15 ml. bulb. The carbon dioxide analyzer is made in similar fashion with a 5 ml. bulb. The fixed-capacity 10 ml. syringes (29, 30, 31) used with the analyzers are calibrated by weighing the water delivered from them.

Constant temperature air or water bath is used for the equilibration. The tonometers are clipped onto a metal drum rotating in the bath.

Van Slyke manometric apparatus with standard equipment is used for the analyses of the liquid phases (3).

PROCEDURE

The liquid to be equilibrated is drawn into the clean dry syringe tonometer through the glass adapter, a needle attached, gas bubbles ejected if present, and the needle plugged with a small rubber stopper. The gas phase is then introduced by injecting measured volumes of the individual gases from syringes through the rubber tubing connection of the tonometer. Alternatively, gas may be drawn into the tonometer by inserting its needle directly into rubber tubing connected to a gas cylinder or gas-filled aspirator bottle. The volume of gas and blood in a 50 ml. syringe should not exceed 50 ml. After the gas phase is introduced, a few drops of mineral oil are placed on the plunger close to the barrel to seal the space between the plunger and barrel. The tonometer is placed in the constant temperature bath.

After equilibration the tonometer is removed from the bath and if any material has dried on the protruding part of the plunger, it is wiped off with a warm wet cloth and more mineral oil is applied.

After rinsing out the needle and adapter by ejecting a little gas, the gas phase is at once removed from contact with the liquid phase by injecting the gas into a gas sample holder (Fig. 1). Then the needle on the tonometer is replaced with a short length of $\frac{1}{8}$ inch bore rubber tubing, all remaining gas is ejected and the tubing stoppered with a glass plug. When whole blood is employed, some mercury is injected into the tonometer at this point in order that the blood may be stirred by shaking before being sampled. Samples of the liquid phase are removed as described by Roughton and Scholander (32). The tonometer is kept in an ice water bath until analyses are completed.

Gas analyses may be carried out in the Haldane apparatus, or with less precision in the Scholander analyzers. In the latter, we prefer to use indigo carmine rather than sodium anthraquinone- β -sulfonate as a catalyst in the alkaline $\text{Na}_2\text{S}_2\text{O}_4$ oxygen absorbing reagent (33). Indigo does not obscure the meniscus and it acts as an oxygen indicator.

The liquid phase is analyzed with the usual apparatus (3).

EXPERIMENTAL RESULTS

The following experiment was designed to compare an accepted procedure (1) with this new technic. Barcroft tonometers and 100 ml. syringes were filled alternately with portions of freshly drawn oxalated

human blood; 25 ml. samples were used in the Barcroft tonometers and 12 ml. samples in the syringes. Portions of the same gas mixture were introduced into the syringes and gas tonometers. The blood and gas phases were equilibrated for an hour at 37–38°C. in an air bath. The gas phases were then separated and stored over mercury. The blood samples were at once analyzed for carbon dioxide and oxygen in duplicate by different analysts. The pH of each sample was promptly measured at 37–38°C. on an L & N potentiometer with a MacInnes glass electrode standardized with 0.05 *M* Bureau of Standards acid potassium phthalate. The gas phases were analyzed in duplicate by different analysts using the Haldane apparatus and the Scholander quick analyzers. The results are given in Table I.

DISCUSSION

In general, comparison of the two equilibration technics reveals no significant differences between the results obtained by one or the other. The agreement between the measured and calculated pH values is equally good in the case of the Barcroft tonometers and the syringe tonometers. This indicates that equilibration of carbon dioxide and the separation of the gas phases is accomplished equally well by either technic. That this is also true for the equilibration of oxygen may be seen by inspection of Fig. 2 which shows that the points on the oxygen dissociation curve obtained by either procedure fall equally close to the oxygen dissociation curve of normal human blood (34). The average gas tensions calculated from the Scholander analyses differed by 0–4 mm. of Hg from those calculated from the Haldane analyses.

The syringe technic has been used in carrying out over two hundred equilibrations of blood and of plasma, and has proved in several respects to be more convenient than the conventional methods: (a) The equipment is less bulky and less expensive. (b) The small size and light weight of the required apparatus makes it more portable. (c) Syringes are easier to use and are more generally available in a variety of sizes than Barcroft tonometers.

We are indebted to Dr. Milan A. Logan, Dr. Shiro Tashiro, and Dr. M. J. Boyd for their help in testing this technic in the instruction of medical students in the Department of Biological Chemistry. Jane K. Friedlander kindly assisted with the blood analyses.

TABLE I
Comparison of the Results of Equilibration of Normal Human Blood in Barcroft and Syringe Tonometers

	Barcroft Tonometer No. 1			Barcroft Tonometer No. 3			Syringe Tonometer No. 2			Syringe Tonometer No. 4		
	Gas		Blood	Gas		Blood	Gas		Blood	Gas		Blood
	Hal- dane	Scho- lander		Hal- dane	Scho- lander		Hal- dane	Scho- lander		Hal- dane	Scho- lander	
Vol. % O ₂	7.81	3.0	10.41	3.82	4.1	9.72	4.08	lost	10.88	3.98	3.6	
	7.86	4.2	10.27	3.85	3.6	9.49	4.12	lost	10.60	3.97	3.4	
Vol. % CO ₂	51.66	5.9	50.65	5.55	5.3	49.55	5.64	5.3	48.40	5.20	5.3	
Measured pH	51.59	6.6	50.25	5.53	5.2	48.97	5.68	lost	47.30	5.12	5.1	
Calculated pH	7.38		7.42			7.43			7.44			
Average % cell		7.39		7.44	7.46		7.41	7.44		7.44	7.45	
% HbO ₂	38.8		38.6			37.6			39.0			
mm. Hg pO ₂	39.4		52.0			48.3			54.1			
mm. Hg pCO ₂	23.8	25		26.8	27		28.7			27.8	24	
	43.3	44		38.7	37		39.6	37		36.1	36	

A portion of this blood saturated with air contained 20.34 vol. % O₂, of which 0.65 vol. % was calculated to be dissolved O₂.

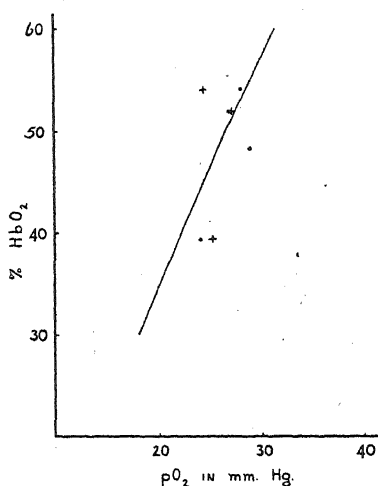


FIG. 2

Comparison of the Normal Oxygen Dissociation Curve of Human Blood at pH 7.40 and 37°C. with Experimentally Determined Points

Circles indicate points calculated from Haldane analyses.

Crosses indicate points calculated from Scholander analyses.

SUMMARY

1. A simple procedure for liquid-gas equilibration is described in which a syringe with adapters is used as a tonometer. The gas phase is stored in a special vessel and analyzed in a Haldane apparatus or in Scholander quick analyzers. The liquid phase is analyzed by the Van Slyke manometric procedure.

2. The apparatus required is relatively inexpensive, is easily obtained or constructed, is portable, is simple and convenient to operate, and is adaptable to varying amounts of liquid and gas.

3. The results obtained by the syringe technic in the equilibration of whole blood are compared with those obtained using Barcroft tonometers, and appear to be equally as good with respect to pH, pO_2 , and per cent oxyhemoglobin, and to agree with the results of other investigators.

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Stereochemical Configuration and Provitamin A Activity

II.* All-*trans*- γ -carotene and Pro- γ -carotene

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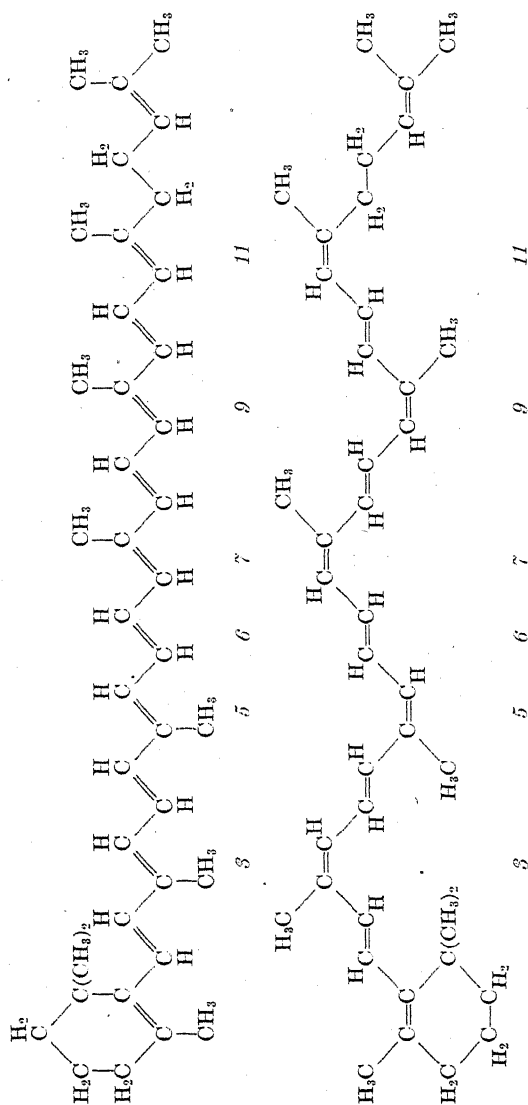
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The preponderant part of the C_{40} -carotenoids which occur in plants evidently possesses all-*trans* configuration. However, a natural pigment, polycopene, $C_{40}H_{56}$, which contains several *cis* double bonds was described three years ago in collaboration with LeRosen, Went, and Pauling (13). A second representative of the same type, pro- γ -carotene, $C_{40}H_{56}$, was then found (10, 9, 7, 8).

On the basis of a study carried out in collaboration with Pauling and LeRosen (12) it is probable that five of the six double bonds in γ -carotene which are available for *trans* \rightarrow *cis* rotations have assumed *cis* configuration in the pro- γ -carotene molecule. Furthermore, it is reasonable to postulate that the only sterically effective double bond in the *trans* configuration occupies a central position in this chromophore as shown by No. 7 in the formula.

That γ -carotene is a provitamin A was first reported by Kuhn and Brockmann (5). Later, these authors, in collaboration with Scheunert and Schieblich (6), indicated that γ -carotene possesses a potency approximately half of that of β -carotene in the rat. We have now investigated to what extent the great stereochemical difference between all-*trans*- γ - and pro- γ -carotene influences this biological activity. Recently it was found (2) that a single *trans* \rightarrow *cis* rotation decreases the activity of β -carotene by about 60%. It seemed possible that the presence of several *cis* double bonds in pro- γ -carotene might destroy this effectiveness.

* For Part I see Reference 2.



Above: All-*trans*- γ -carotene (ordinary γ -carotene).

Below: Pro- γ -carotene (3,5,7,9,11-penta-*cis*- γ -carotene). (The double bonds which can undergo *cis-trans* rotations are numbered in both formulas. For details of nomenclature see (8).)

Tests are also reported of the inactivity of polycopene which was to be expected on the basis of the negative behavior of ordinary (all-*trans*-) lycopene (3).

EXPERIMENTAL

The pro- γ -carotene employed was isolated from *Pyraacantha* and the γ -carotene from *Mimulus* as described earlier (10, 11). The polycopene sample was obtained by Dr. A. L. LeRosen from Tangerine tomatoes (13). Both formed chromatographically homogeneous crystals which were kept sealed under CO₂. Spectrophotometric and chromatographic tests proved that steric or other changes occur to an extent of less than 6.5% with pro- γ -carotene in Wesson oil in the presence of α -tocopherol for about 3 days. After the conclusion of the bioassays, the remaining pigments were shown to have retained their chromatographic homogeneity. Furthermore, the molecular extinction coefficient of γ -carotene in hexane was then found to be 14.4×10^4 at 462 m μ while the best published value (12) is 14.6×10^4 . The other materials and procedures were described earlier (2). The rats employed were from our own stock colony. When they had exhibited symptoms of A-deficiency, such as constant weight for 5 days and xerophthalmia, they were assigned to one of ten groups. These each received, in addition to the A-free diet, one of the following supplements daily in 0.1 ml. of Wesson oil containing 0.5 mg. of α -tocopherol, over a 28-day period: 0.6 or 1.2 μ g. of β -carotene; 0.6, 1.2, or 2.4 μ g. of γ -carotene; 0.6, 1.2, 2.4, or 4.8 μ g. of pro- γ -carotene; and 60 μ g. of polycopene; and also oil-tocopherol alone (negative controls).

Table I gives a summary of the results obtained on the 169 rats used.

The dosage/gain in weight curves are given in Fig. 1. The points for the three lower dosages for pro- γ -carotene fall on a straight line which is reasonably parallel to that of β -carotene. With γ -carotene the curve for the two higher doses also is parallel to that of β -carotene. The potency of the pro- γ -carotene was calculated, by the method of Coward (1), to be 2.3 μ g. (average of 2.1 and 2.5 μ g.) as biologically equivalent to 1.0 μ g. of β -carotene. On the other hand, 3.6 μ g. of γ -carotene were required to equal the biological potency of 1.0 μ g. of β -carotene.

DISCUSSION

Pro- γ -carotene has been found to be a powerful provitamin A in the rat with an activity which averages 44% of that of β -carotene. Since some of the fruits mentioned contain substantial amounts of pro- γ -carotene (*e.g.* 20 mg. per kilo of fresh *Pyraacantha* berries), this pigment can well contribute to the vitamin A requirements of some birds and,

TABLE I
Summary Table of Bioassay Experiments on Male and Female Rats Receiving β -Carotene, γ -Carotene, Pro- γ -carotene, or Prolycopene

The average results on males and females are weighted equally. Where animals died during the course of the experiments, the numbers of animals still alive which are included in the average is given in parentheses. The average age at the start of the depletion period was 23 days.

Supplement	Dose per day	Number of rats		Depletion period			Assay period						
				Average weight at start	Average duration	Average final weight	Average increase in body weight on following days						
		Male	Female	$\mu g.$	days	$\mu g.$	5th	10th	15th	20th	25th	28th	Average final weight
β -Carotene	0.6	7	8	41.5	24	88.8	1.0	10.0	16.4	19.9	23.4	25.0	$\mu g.$ 114.0
	1.2	8	8	42.0	22	91.2	3.3	18.8	27.0	38.2	44.7	47.9	139.1
γ -Carotene	0.6	8	8	41.4	23	89.6	-0.4	6.6	0.8 (15)	-0.7 (14)	-1.7 (10)	-5.4 (10)	84.4
	1.2	8	8	41.0	23	90.8	-3.4	1.4	0.7 (14)	-0.7 (14)	2.7 (10)	1.0 (9)	93.2
Pro- γ -carotene	2.4	7	7	42.0	23	90.2	3.6	11.5	17.1	21.1	25.5	28.2	118.4
	0.6	6	6	41.2	22	89.2	0.5	4.0	4.0	1.9	0.3	0.2	89.4
	1.2	6	6	41.5	24	89.6	2.0	7.9	8.3	12.4	21.6	23.4	112.4
	2.4	6	6	41.4	22	89.3	5.1	13.7	20.3	24.7	35.8 (11)	40.0 (11)	128.2
Prolycopene	4.8	6	6	42.6	24	89.7	2.7	18.0	24.9	34.8	42.3	47.6	137.3
	60.0	5	5	40.8	23	89.7	-6.6 (9)	-8.4 (8)	-8.3 (6)	-11.0 (4)	1.0 (1)	-3.0 (1)	83.0
Negative controls	0.0	18	16	41.5	23	89.0	-6.0 (33)	-7.4 (25)	-9.3 (21)	-13.2 (16)	-14.0 (8)	-14.2 (6)	78.2

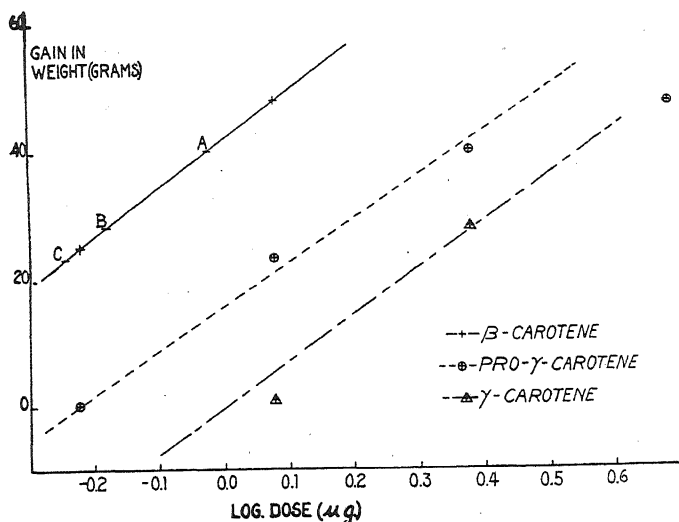


FIG. 1

Relationship of Gain in Weight to Dosage of β -Carotene, Pro- γ -carotene, and γ -Carotene

Points A and C represent the projection of the average gain in weight for the groups receiving 2.4 and 1.2 μ g. of pro- γ -carotene respectively on the β -carotene curve. Point B represents the projection of the average gain in weight of the group receiving 2.4 μ g. of γ -carotene on the β -carotene curve.

possibly, of some other animals. In contrast, the palm fruits mentioned which are also available for human nutrition only contain as little as 1 mg. of pro- γ -carotene/kilogram of fresh material.

The effect of ordinary γ -carotene observed in the present study is considerably weaker than has been reported by other authors. We cannot state yet whether or not our result was influenced by the fact that the analytically pure γ -carotene sample (*ex Mimulus*) employed here (as well as a sample prepared from pro- γ -carotene by iodine catalysis) has a very different melting point from some other samples obtained from commercial carotene (5, 11).

Kuhn, Brockmann, Scheunert, and Schieblich (6) carried out their important study in order to find an experimental check for the assumption that, on the basis of the structural formulas, the β -carotene molecule can be expected to give rise to two molecules of vitamin A in the

rat while the corresponding figure will be one for α - or γ -carotene. These authors were successful in demonstrating that the limiting dose ("Grenzdosis") of α - or γ -carotene (5 μ g.) was as a matter of fact the double of the necessary β -carotene dose (2.5 μ g.). This "Grenzdosis," *i.e.* the necessary daily dose which enabled at least 70% of the animals to increase their weight by at least 10 g. during 35 days, was not claimed to be a means for an accurate estimation of the relative efficiencies of the carotenes. Indeed, the "Grenzdosis" of β -carotene produced the desired response not in 70% but in 100% of the animals employed; and it may well have exceeded the true "Grenzdosis" by an unknown amount. It seems, therefore, that the data published by Kuhn *et al.* point rather to a lower efficiency of γ -carotene than 50% of that of β -carotene.

The more potent response of β - and γ -carotene in the present tests may be because a different strain of rats was employed and, especially, because α -tocopherol was added, which synergistically increases the effectiveness of the carotenes (4).

The mechanism of the conversion of pro- γ -carotene into vitamin A in the body is as yet unknown. The strength of the pro- γ -carotene effect in comparison with that of all-*trans*- γ -carotene would suggest that the former pigment can be converted into vitamin A without the intermediate formation of the latter. Perhaps a direct enzymatic action is made possible by the general shape of the pro- γ -carotene molecule which is almost as straight as that of all-*trans*- γ -carotene. Of course, this interpretation would postulate that the respective rates of absorption and destruction are of the same order for both stereoisomers.

SUMMARY

Pro- γ -carotene, $C_{40}H_{56}$, a naturally occurring stereoisomer of γ -carotene, is a powerful provitamin A whose biological effect in the rat was found to be 44% of that of β -carotene; γ -carotene (*ex Mimulus*) showed a potency of only 28% of that of β -carotene. As expected, prolycopene, $C_{40}H_{56}$, is inactive even in daily doses of 60 μ g.

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A Lysineless Mutant of *Neurospora* and Its Inhibition by Arginine

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INTRODUCTION

It has been shown that rats, dogs, and human beings require certain amino acids in their diets (1, 2, 3, 4). In contrast to the higher animals the wild type strains of the mold *Neurospora* thus far collected in nature do not require amino acids as nutrients, for they are able to grow on an aqueous medium to which only inorganic salts, a carbon source, and biotin are added. In the laboratory, however, it has been possible to obtain mutant strains of the mold which do require individual amino acids including several of those indispensable to the rat. Beadle and Tatum (5) have induced gene mutations of this nature by means of X-rays and ultraviolet light, and among the amino acid mutants so obtained a number fail to grow unless the basal medium is supplemented with lysine. It is the purpose of this paper to describe one of these lysineless mutants, No. 4545.

Genetic Analysis

Genetic analysis in *Neurospora* is based on the isolation of ascospores. The techniques and interpretation have previously been described by Dodge (6) and Lindegren (7). The method of identifying genes concerned in particular biochemical reactions in *Neurospora* has been described by Beadle and Tatum (5).

That strain No. 4545 differs from the wild type as the result of a gene mutation is borne out by the data derived from outcrosses. A cross was made between the normal stock, No. 25 *a*, and the lysineless mutant, No. 4545. From this cross the spores of seventeen

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asci were isolated, the position of each spore in the ascus being recorded. After the spores had germinated the resultant strains were tested for their ability to grow without lysine; if they were able to grow on the unsupplemented basal medium they were classified as *normal*, otherwise as *lysineless*. In all cases no more than four normal spores were found per ascus and no more than four lysineless. Furthermore, the normal spores were always in pairs in the ascus as were the lysineless spores. From these data it is clear that a genetic mutation differentiates this lysineless strain from the wild type. Since no more than four spores of either type were found per ascus, it is probable that only one gene is involved.

With regard to the location of this gene on the chromosomes, the above mentioned asci indicate that it is probably thirty or more units from the centromere since in eleven of the seventeen, segregation took place in the second meiotic division. In order to secure more information regarding the gene's location, another cross was made, 4637 *a* by 4545 *A* (albino-1 by lysineless). From this cross thirty asci were dissected and the spores tested as before. The results are summarized in Table I. It had previously been established in this laboratory that

TABLE I

Genetic Data Showing Linkage of Lysineless, Albino, and Sex

Summary of asci obtained from the cross 4637-*a* by 4545-*A*. The symbols *A* and *a* refer to the mating type, *lys* to lysineless (4545), and *al* to albino-1 (4637). The symbol + indicates the normal allele of the mutants.

Ascus frequency	Spore pair			
	I	II	III	IV
23	<i>A</i> + <i>lys</i>	<i>A</i> + <i>lys</i>	<i>a al</i> +	<i>a al</i> +
2	<i>A</i> + <i>lys</i>	<i>A</i> + +	<i>a al lys</i>	<i>a al</i> +
1	<i>A</i> + <i>lys</i>	<i>A al</i> +	<i>a</i> + <i>lys</i>	<i>a al</i> +
1	<i>A al</i> +	<i>a al</i> +	<i>A</i> + <i>lys</i>	<i>a al lys</i>
1	<i>A al</i> +	<i>A al</i> +	<i>a</i> + <i>lys</i>	<i>a</i> + <i>lys</i>
1	<i>A</i> + +	<i>A</i> + +	<i>a al lys</i>	<i>a al lys</i>
1	<i>A</i> + +	<i>A al</i> +	<i>a al lys</i>	<i>a</i> + <i>lys</i>

a linkage exists between the albino-1 and sex loci, and that they are located on opposite sides of the centromere. From the results given in the table it is concluded that the lysineless gene is also located on the first chromosome. The order of genes is as follows:

sex—centromere—albino-1—lysineless.

Since these data were secured it has been found that strain 4637 gives reduced crossover values in a region of its chromosome. Therefore the map distances calculated from the table are not reliable, but the order nevertheless appears to be correct.

Growth Experiments

The growth experiments reported here were carried out in either of two ways. The first involves growing the mold in liquid culture and weighing the resultant mycelium after drying. The composition of the basal medium used for this method is described by Horowitz and Beadle (8). All experiments described were carried out in 250 ml. Erlenmeyer flasks using 25 ml. of basal medium per flask. After inoculation with a drop of conidial suspension, the cultures were incubated at 25°. The time of incubation varied with the nature of the experiment and is given with experiment described. Flasks were shaken twice daily to prevent excess sporulation.

A supplementary method of measuring the growth response quantitatively is to use the tube method (9), which gives a measure of the rate of advance of a mycelial frontier over an agar surface. The basal medium used is the same as in flasks, but 3% agar is incorporated into it and 15 ml. are used per 10–12 inch growth tube.

TABLE II

Effect on Growth of Other Amino Acids in the Presence of Lysine

Dry weight obtained on a standard concentration of lysine to which 3 mg. of each of the listed substances have been added individually. None of these supported growth in the absence of lysine. Time of incubation was 168 hours.

Substance added	Dry wgt. mg.	Substance added	Dry wgt. mg.
Control (without lysine)	00.0	<i>dl</i> - α -Methyl-valine	39.1
Control (with lysine)	36.6	<i>l</i> -Asparagine	39.2*
Glycine†	35.6	<i>l</i> -Glutamic acid	40.0*
<i>dl</i> -Alanine	38.6	<i>l</i> -Glutamine	39.0
<i>dl</i> -Serine	41.4*	<i>l</i> -Cystine	37.8
<i>dl</i> - α -Aminobutyric acid	35.8	<i>dl</i> -Methionine	38.0
<i>dl</i> -Threonine	38.4	<i>l</i> -Arginine	00.0*
<i>dl</i> -Norvaline	35.2	<i>dl</i> -Citrulline	38.9
<i>dl</i> - α -Amino- α -methyl-butyric acid	37.0	<i>l</i> -Histidine	37.5
<i>dl</i> -Ornithine	38.1	<i>dl</i> -Phenylalanine	36.8
<i>dl</i> -Leucine	37.6	<i>dl</i> -Tryptophan	33.4*
<i>dl</i> -Isoleucine	43.8*	<i>dl</i> -Tyrosine	38.3
<i>dl</i> -Norleucine	33.8*	<i>dl</i> -Proline	36.7
<i>dl</i> - ϵ -Amino- <i>n</i> -caproic acid	36.2	<i>dl</i> -Hydroxyproline	37.7
<i>dl</i> -Valine	39.6*	Urea	37.5

* Statistically significant deviations from the mean of the ten lysine controls (36.6 mg.). The standard error of the difference is 1.32 where the standard deviation is calculated from the lysine controls, and assuming that the standard deviation does not vary over the range of the experiment.

† All figures except lysine controls are an average of two determinations.

To establish that the gene in question prevents the synthesis of lysine, three lines of evidence can be advanced:

(1) For growth, mutant No. 4545 requires lysine in the basal medium. Of twenty-eight amino acids tested individually (those listed in Table II) only lysine was found to initiate growth.

(2) Upon addition of sufficient lysine to the basal medium the mutant will grow at a rate approximating that of the wild type, *i.e.*, at the rate of the non-mutated strain. This was tested by both methods described above. The tube method showed that 4545 grew at a rate of 3.55 mm. per hour when an optimum amount of *dl*-lysine

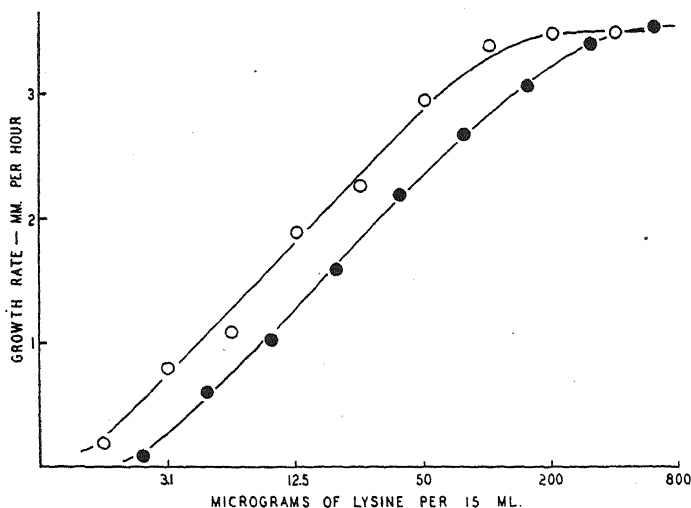


FIG. 1

Rate of Growth Over an Agar Surface

Open circles represent growth response to *l*(+) lysine and solid circles growth response to *dl*-lysine. Rates are averages of two determinations.

was added to the medium and at a rate of 3.48 mm. per hour when enough *l*(+) lysine was added. The rate of the normal control on a similar medium was 3.58 mm. per hour. Fig. 1 shows the result of such an experiment.

Using dry weight as a measure of growth, the mutant again approximated the growth of the normal control. This is apparent from the results summarized in Table III. After 92 hours' incubation the mu-

tant had attained a dry weight of 70.0 mg. on 25 ml. of medium containing 14.6 mg. of *l*(+) lysine, HCl; the wild type weight was 73.9 mg. on an identical medium.

(3) When the lysine is exhausted from the medium, growth of the mutant ceases. This fact was established by adding enough *l*(+) lysine, HCl, 0.15 mg. per flask, to the basal medium to produce approximately 8 mg. dry weight of mycelium. Cultures were set up in duplicate, inoculated, and incubated for seven days. At the end of this time the mycelium was removed, and to one of a pair of flasks

TABLE III

Growth at Various Concentrations of Lysine

<i>l</i> (+) Lysine, HCl added per flask	Dry weight after 92 hours*
mg.	mg.
0.00	0.0
0.23	9.4
0.46	17.8
0.91	29.2
1.82	39.9
3.65	63.1
7.30	71.2
14.6	70.0
29.2	64.1
Wild type No. 1-A	
14.6	73.9

* Ave. of two determinations.

more lysine was added. After autoclaving and reinoculation with the mutant, it was found that the medium to which additional lysine had been added supported growth while the other did not.

Upon establishing the fact that the synthesis of lysine is prevented in strain 4545, the question arises: Is the requirement specific for *l*(+) lysine as is the case in rats and mice (10, 11) or can this *Neurospora* mutant use the unnatural form of lysine? Since no *d*(-) lysine was readily available, and since it is possible to make accurate growth comparisons with so simple an organism, the activity of *l*(+) lysine was compared to that of the racemic mixture. Fig. 1 shows the rates observed in growth tubes on various concentrations of natural and racemic lysine. The curves show that *l*(+) lysine is twice as active as *dl*-lysine.

Since *Neurospora* is known to have a *d*-amino oxidase which will slowly oxidize *d*(-) lysine (12), it seemed possible that this mutant might nevertheless be able to utilize the unnatural isomer through oxidative deamination and subsequent reamination. These reactions might be somewhat slow and not be reflected in a rate measurement where the mycelium is constantly progressing over a surface of fresh medium containing *l*(+) lysine. To check this possibility the activities of natural and racemic lysine were compared in liquid culture so that

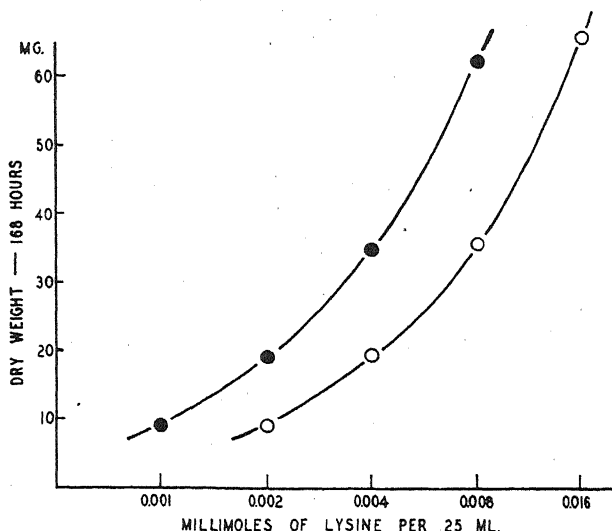


FIG. 2

Total Growth in Liquid Culture

Solid circles represent the dry weight from *l*(+) lysine and open circles that from *dl*-lysine. Points represent averages of two determinations.

the total dry weight resulting from various amounts of lysine could be determined. This was done by growing the mold for seven days on a medium containing one mg. per ml. of both asparagine and glutamic acid in addition to varying amounts of lysine. The reason for adding these two substances is discussed later. From other extensive experiments it is known that, under the conditions of these experiments, no appreciable increase in dry weight would be obtained by incubating longer than seven days. Fig. 2 shows the results of this experiment,

and it is again apparent that this strain did not make appreciable use of the unnatural isomer. These experiments, measuring both absolute rate and total growth, clearly indicate that 4545 cannot utilize $d(-)$ lysine, and it seems probable that it cannot use the keto acid analogue either.

In the course of experiments preliminary to the investigation of a lysine bioassay method utilizing this mutant, it was necessary to find out whether other amino acids would stimulate or retard growth when lysine is present in the medium. It has been shown by D. M. Bonner (unpublished) that the dry weight of the normal strain after three days' incubation is almost doubled by the addition of asparagine or aspartic acid to the basal medium. This suggests that available amino nitrogen is a limiting factor in the medium, and that when lysine is supplied, some of it may be used as a source of amino nitrogen and thus be wasted so far as the lysineless mutant is concerned.

The results of an experiment designed to test for stimulation or inhibition by other amino acids in the presence of lysine are given in Table III. Flasks containing a standard concentration of $l(+)$ lysine, HCl, 29.2 μ g. per ml., were individually supplemented with 3 mg. of the substances listed in the table, and were then inoculated and incubated for seven days to insure complete utilization of the lysine. Statistically significant variations were noted in eight cases, of which five were cases of a stimulatory effect. More extensive experiments showed that a number of other amino acids tested in this experiment also show a tendency to stimulate when used in higher concentrations. Such an effect in this mutant, which under all other conditions seems to be strictly limited by the available lysine, is interpreted as a sparing action on lysine when an additional source of amino nitrogen is added to the medium. This hypothesis is in harmony with the work of Schoenheimer and his colleagues (13, 14) on mice showing that, in transamination reactions, lysine acts as a donor of amino groups, but that the oxidation product does not act as a recipient. In the lysineless mutant of *Neurospora* described here the stimulatory amino acids might exercise a sparing effect on lysine by preventing its irreversible deamination.

Because of this stimulatory action, both asparagine and glutamic acid were added to the culture medium in the experiment on which Fig. 2 is based. Like the other amino acids not essential to the growth of this mutant, $d(-)$ lysine has a stimulatory effect which can be

replaced by that of asparagine and glutamic acid. The latter two are apparently better sources of amino nitrogen than *d*(-) lysine.

Inhibition by Arginine

In the experiment described above, eight cases of significant variation were noted when various amino acids were added to the medium containing lysine. Three of these were instances of inhibition. When considered from the viewpoint of establishing a bioassay method for lysine, the effects of tryptophan and norleucine are negligible because the former is broken down in acid hydrolysis and the latter is present in proteins only in small quantities. The effect of arginine, however, which prevented growth completely, was investigated further. These experiments showed that the critical factor which determines this inhibition is not the absolute amount of arginine present but rather the molecular ratio of arginine to lysine. This fact was established by an experiment in which 250 ml. of basal medium containing a given amount of lysine was distributed equally among ten flasks. This was done at five concentrations of lysine making five series of ten flasks each. An eight-fold range of concentrations was covered. To the flasks of each series varying amounts of *l*(+) arginine were added. These cultures were incubated for 96 hours, and thereafter the mycelial pads were dried and weighed. Fig. 3 shows the results. From the curves are calculated the molecular ratios of arginine to lysine which reduced the dry weight to one half that obtained from the arginine-free control flask of each series. This ratio is hereafter referred to as A/L. The curve from the series containing 0.0025 millimole of lysine per flask gave A/L equal to 1.2, the rest being 1.1, 1.0, 1.0, and 1.0 respectively. It is therefore evident that a molecular ratio of arginine to lysine of approximately one will reduce growth to about one half.

Of the two optical isomers of lysine only the naturally occurring one is effective in relieving the inhibition, and only the natural form of arginine is capable of bringing it about. An experiment similar to the one above was set up, but here the four possible combinations of natural and racemic lysine against natural and racemic arginine were made. Only one series was run for each combination. A/L was again calculated and the values obtained are given in Table IV-A. It is concluded from a comparison of these values that only the natural optical isomers are involved.

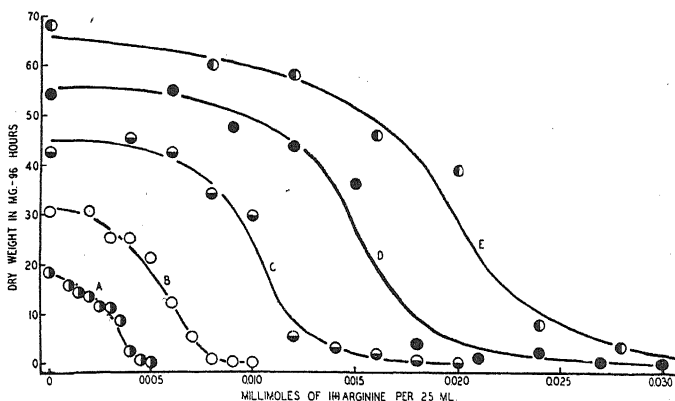


FIG. 3

Growth at Various Concentrations and Ratios of Arginine and Lysine

A—0.0025 millimoles of *l*(+) lysine per 25 ml.

B—0.0050 millimoles of *l*(+) lysine per 25 ml.

C—0.0100 millimoles of *l*(+) lysine per 25 ml.

D—0.0150 millimoles of *l*(+) lysine per 25 ml.

E—0.0200 millimoles of *l*(+) lysine per 25 ml.

Three points at higher concentrations of arginine on curve E are not shown in the figure. They show that this curve also drops to zero. Each point in the figure represents a single determination.

TABLE IV

Factors Affecting Arginine Inhibition in Strain No. 4545

A/L is the molecular ratio of arginine to lysine which reduced growth to one half of that in the arginine-free control flask. Cultures in part A were incubated for 96 hours. Those in part B were incubated for 72 hours.

A—Optical Isomerism			B—Hydrogen Ion Concentration	
Arginine	Lysine	A/L	pH	A/L
<i>l</i>	<i>dl</i>	0.55	4.8	0.95
<i>l</i>	<i>l</i>	1.13	5.9	0.82
<i>dl</i>	<i>dl</i>	1.00	6.7	0.58
<i>dl</i>	<i>l</i>	2.40	7.2	0.48

In an experiment to discover the effect of pH on this inhibition, it was found that the inhibition ratio, A/L, falls as the hydrogen ion concentration is decreased. This effect is shown in Table IV-B, which

is based on an experiment in which A/L values were calculated from four series of flasks which were buffered at four pH levels with $M/20$ phosphate buffer.

The wild type strain of *Neurospora*, which can make enough lysine to supply its needs, is not inhibited by arginine even at a level of $0.095 M$ (2% $l(+)$ arginine, HCl). Even when the concentration of arginine was brought to $0.475 M$ the normal strain grew after an initial lag of 36 hours. This lag could probably be explained by the need of the mold to adjust itself to the high osmotic pressure of a medium containing 10% arginine, HCl.

Arginine inhibition is not due to a gene separate from the one concerned in the synthesis of lysine since thirty separately occurring lysineless strains, known to be of at least three genetic types, all show the phenomenon. For the same reason it can be said that it is not due to the mutation of a particular gene in the synthesis. Rather it must be concluded that the effect is inherent in the utilization of lysine. In this regard the wild type would be expected to be identical with the mutant. How the wild type may possibly circumvent this inhibition is discussed below.

DISCUSSION

The inhibitory effect of amino acids has been noted in many instances. Gordon and McLeod (15) found cystine, glycine, phenylalanine, and tryptophan inhibitory to various cocci. They suggested that deamination products might be the actual inhibitors. Sullivan, Hess, and Sebrell (16) found that high percentages of cystine, tyrosine, tryptophan, and lysine were decidedly injurious to rats. Nielsen and Hartelius (17) showed *Saccharomyces cerevisiae* to be inhibited by β -alanine, and that this inhibition could be relieved by addition of asparagine and aspartic acid to the medium. In experiments on *B. anthracis* Gladstone (18) describes instances of competitive inhibition of the amino acids for enzymes. Here leucine, threonine, and α -amino-butyric acid inhibitions were released by valine, and valine inhibition was released by the former three. Also isoleucine, norleucine, and serine toxicity was released by valine and leucine; and serine inhibition could be relieved by threonine.

One hypothesis to explain the known facts about arginine inhibition in *Neurospora* depends on the suggestions of Linderström-Lang (19)

and Kalekar (20) that peptides may not be formed through the amino acids, but through corresponding aldehydes. If, in the mutant, lysine is converted to an energy-rich compound before peptide formation, then arginine may compete with lysine in this conversion. In the normal strain, synthesis of a lysyl peptide would then not necessarily involve lysine, and this would account for the fact that the normal strain is not inhibited by arginine.

SUMMARY

Genetic evidence indicates that strain No. 4545 of *Neurospora crassa* differs from the normal strain in a single gene which is located on the first chromosome. Growth experiments show that, in addition to the nutrient requirements of the wild type, strain No. 4545 needs only the amino acid lysine for normal growth. Only the natural optical isomer of lysine is active in promoting growth.

The mutant is specifically inhibited when the molecular ratio of arginine to lysine approaches a critical value. A molecular ratio of approximately one, under the usual cultural conditions, reduces the growth to one half of that in the arginine-free control culture. If this ratio is doubled, growth is completely inhibited. Only the natural optical isomers of the two amino acids are active in these respects. Arginine does not inhibit the non-mutated wild type, which grows normally in the presence of high concentrations of arginine.

The author wishes to express his appreciation to the members of the genetics laboratories of Stanford University for providing the mutant and for helpful advice and suggestions.

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Spontaneous Hydrolysis of Diphtheria Antitoxic Pseudoglobulin

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INTRODUCTION

The present paper reports a study of an aged sample of diphtheria antitoxic pseudoglobulin which was found to have spontaneously undergone partial hydrolysis and to contain a new antitoxic fraction similar to that artificially obtained by the action of proteolytic enzymes. While the sample studied here in detail had been kept for a period of twelve years, several other eight- to nine-year-old samples that were examined evidenced the same changes and suggested that the phenomenon was a common occurrence in old pseudoglobulin preparations.

MATERIALS AND METHODS

Antitoxic pseudoglobulin Lot No. 626A. The pseudoglobulin from several pooled antitoxic horse sera was separated in 1931 by fractional ammonium sulfate precipitation; 0.3% of cresol was added, and the material was stored in the cold. In 1934, the residual euglobulin was removed from this preparation by iso-electric point precipitation (1). The finished product, which contained 150 mg. of protein, 9 mg. of NaCl, 3 mg. of cresol, and 1920 Lf per ml., was again stored in the cold until 1941 when it was distributed for therapeutic purposes. Two years later, the unused material that had been returned to this laboratory was pooled and employed in this study. The Lf value at that time was 1700 units per ml.

Diphtheria toxin. The toxin was produced by growing a culture of *Corynebacterium diphtheriae*, No. 5 (Park-Williams No. 8), on semisynthetic medium (2). Its Lf value was obtained by titration against a standard serum. When necessary, it was concentrated by pervaporation followed by dialysis against phosphate buffer.

Protein determinations. The various antitoxic solutions were dialyzed against running water and their total concentration determined with the Zeiss dipping refractometer. The value of 0.00185 was taken for the specific refractive increment of globulin (3), which corresponds to 0.207% concentration per scale division.

Antitoxin titrations. The Lf values were determined in a water bath at 50°C. by the constant antigen method in constant fluid volume (usually 1.0 ml.). Little difference was observed between constant antigen and constant antibody titrations.

Floccule nitrogen. Toxin-antitoxin mixtures were placed at 50°C. for from 1 to 3 hours and left overnight in the cold. The floccules were centrifuged twice and washed with chilled saline and their nitrogen content determined by Kjeldahl analysis.

Copper. The preparations containing copper were dry-ashed at 600°C., the ash taken up in dilute HCl, and the copper determined by the method of Biazzo, adapted by Elvehjem and Lindow (4).

Osmotic pressure determinations. These were made by the author's method (5) on protein fractions in saline or dilute phosphate buffer, pH 6.8. Determinations were made in duplicate, and usually checked within 5 per cent or less.

Sedimentation rates. These were kindly determined by Dr. A. Rothen at the Rockefeller Institute for Medical Research, New York City.

RESULTS

Physical properties of diphtheria antitoxin No. 626A. The pseudoglobulin did not appear to differ from other similar preparations until it was accidentally observed that the addition of a 5.0% copper sulfate solution failed to produce a precipitate unless the pseudoglobulin was first dialyzed against tap water. Dialysis caused a loss of 9.0% of the dry weight—exclusive of NaCl. On the other hand, precipitation of the undialyzed pseudoglobulin with trichloroacetic acid left 9.4% of the total nitrogen in solution. It was inferred that at least 9.0% of the original protein had broken down to the amino acid or polypeptide stage.

When the addition of a 5.0% solution of copper sulfate to an equal volume of the pseudoglobulin was followed by dialysis against tap water (total solids 0.01%, pH 9), most of the protein precipitated. About 20 per cent of it, however, remained in solution. This soluble fraction possessed properties decidedly different from the original material, the most obvious being a decrease in ratio of protein to antitoxin and a considerable shortening of the flocculation time. Subsequent work showed that the results were not appreciably affected by varying the copper sulfate concentration, the temperature, or the time elapsing between mixing and dialysis. Furthermore, an antitoxic fraction endowed with the same properties was obtained when copper sulfate was replaced by other metal salts, such as ZnSO_4 , HgCl_2 , or AlCl_3 . It thus appeared that lot No. 626A contained a protein fraction that was particularly resistant to denaturation by heavy metals. The observation that this fraction was also resistant to acid denatura-

tion offered another means of separating it. Two convenient methods for the preparation of this fraction are as follows:

Method 1. Three volumes of a 2.0% solution of CuSO_4 were added to one volume of the antitoxic pseudoglobulin. The mixture was dialyzed in cellophane against running tap water for 48 hours, and the precipitate filtered off. When dialyzed against distilled instead of tap water, the solution became opalescent but the denatured protein did not precipitate.

Method 2. Two volumes of water were added to one volume of the antitoxic pseudoglobulin. The solution was heated to 50°C ., acidified with $N/10$ HCl to pH 3.5, held at this temperature for 15 minutes, and neutralized with disodium phosphate. The denatured protein was removed by one-third saturation with ammonium sulfate and discarded.

Both solutions as finally obtained in water were clear and stable. The solution obtained by the copper method was slightly blue and contained 0.006 mg. of copper per mg. of protein. This could be removed to a great extent by dialysis against buffer solutions or by ammonium sulfate precipitation. The protein isolated by either method was soluble in distilled water; in about 1.0 per cent concentration, it required 44% ammonium sulfate saturation to initiate precipitation, as opposed to 33% for the original, and from 55 to 60% saturation for complete precipitation.

The mean molecular weight of lot No. 626A, calculated from osmotic pressure measurements, was 129,000, or appreciably below the weight of about 163,000 found for recently prepared material.* The resistant fraction from lot No. 626A gave weights ranging from 98,000 to 117,000, depending on the preparation studied (Table I).

Results from sedimentation measurements are given in Table II. At 0.54% concentration, lot No. 626A appeared fairly homogeneous, with a sedimentation constant of about 6.1×10^{-13} [the sedimentation constant of native pseudoglobulin is about 7.0×10^{-13} (10)]. At a slightly higher concentration, the main component had a constant of 5.95, while a second component appeared that represented roughly

* The method permits the rapid determination of pressures in protein concentration sufficiently low (from 0.5 to 1.0%) to avoid significant deviation from van't Hoff's law. The same approximate value of 163,000 was found for fresh human globulin (6), while the values carefully obtained for unfractionated horse globulin by Adair and Robinson (7), and by Burk (8) are on the average slightly higher (175,000 and 173,000 respectively). These authors used a more cumbersome method which necessitates extrapolation to infinite dilution of the specific pressures obtained in various concentrations.

TABLE I

Nitrogen : Antitoxin Ratios and Molecular Weights of Various Diphtheria Antitoxin Preparations

Antitoxin	mg. antitoxin nitrogen per Lf		Molecular weight (by osmotic pressure)
	In whole solution*	In flocclules	
Antitoxin Lot No. 626A (12 years old)	.0114	.00124	129,000
Split fraction (Cu) Prep. 1	.0065	{.00100† .00117	114,000
Split fraction (Cu) Prep. 2	.0080†		102,000
Split fraction (Cu) Prep. 3	.0063	.00115	117,000
Split fraction (HCl) Prep. 4	.0063	.00119	98,000
Split fraction (HCl) Prep. 5	.0068	.00118	
Split fraction (HCl) Prep. 6	.0065	.00116	103,000
Average of Preparations 1 to 6	.0065	.00117	107,000
Antitoxin Lot No. 992 (6 months old)		.00154	164,000
Antitoxin Lot No. 998 (2 months old)		.00165	162,000

* On the assumption of a protein nitrogen content of 14.3% (9).

† Not included in average. In this case dialysis took place for three days at 18°C. instead of 4°C., which may explain the loss in antitoxic potency.

‡ Not included in average.

TABLE II

Sedimentation Rates of Diphtheria Antitoxic Pseudoglobulin Lot No. 626A*

Pseudo- globulin	Concen- tration %	Temper- ature °C.	$S_{T}^{saline} \times 10^{13}$	$S_{20}^{\circ} \times 10^{13}$	Remarks
Whole solution	0.54	24.8.	6.83	6.0	Fairly homogeneous
Whole solution	0.54	25.0 ₅	7.18	6.2	Fairly homogeneous
Whole solution	0.90	25.3	6.95	5.9 ₅	Second component = ca. 30% of total with S_{20}° = ca. 4.7 $\times 10^{-13}$
Cu fraction (Prep. 1)	0.60	25.4	5.92	5.1	Very homogeneous

* Results reported by Dr. A. Rothen, Rockefeller Institute for Medical Research, New York City.

30% of the total and had a constant of about 4.7. The resistant fraction alone, on the other hand, was very homogeneous, with a constant of 5.1 at 0.6%.

The following records some of the antitoxic properties of this re-

sistant, low-molecular-weight fraction. For the sake of convenience, it is referred to as "split" antitoxin.

Serologic properties. Constant antigen and constant antibody titration of diphtheria toxin and antitoxin give, in general, almost identical results (11, 12), a fact that was also observed here with fresh pseudoglobulin preparations. The titration of the split product from lot No. 626A is given in detail in Table III. It will be seen that in constant antibody titration there was a slight shift of optimal proportions toward toxin excess, the direction in which it would have been expected.

TABLE III

Constant Antigen and Constant Antibody Titration of Split Diphtheria Antitoxic Pseudoglobulin

Flocculation time in minutes at 50°C.

Pseudoglobulin solution (ml.) added to 25 Lf of toxin	0.125	0.15	0.20	0.25	0.30	0.40	0.50
				minutes			
Total volume—1.0 ml.	65	33	8	6	8	20	47
Total volume—2.0 ml.	120	56	27	19	23	43	110
Toxin (Lf) added to 0.25 ml. of pseudoglobulin solution	50	40	30	25	20	15	12.5
				minutes			
Total volume—1.0 ml.	40	11	5	6	13	55	120
Total volume—2.0 ml.	53	23	14	17	25	85	>120

The flocculation time of the split antitoxin at various toxin concentrations was compared with that of the original material. The toxin and antitoxin were dissolved in 0.05 *M* phosphate buffer, pH 6.8, and mixed in optimal constant antigen proportions. Table IV shows that, whereas the flocculation time of the whole untreated solution of antitoxic pseudoglobulin was about that of other similar preparations, the split antitoxin flocculated about seven times as fast.

TABLE IV

Optimal Ratio Flocculation Time of Diphtheria Antitoxic Pseudoglobulin Lot No. 626A and of Split Antitoxin

Concentration (Lf per ml.)	5	10	15	20	25	45
			minutes at 50°C.			
Lot No. 626A		210	130	80	60	25
Split fraction	52	28	19	12	7	

Whereas the whole product, lot No. 626A, contained 0.0114 mg. of protein nitrogen per Lf, the split fraction separated from it contained on an average 0.0065 mg. of nitrogen per Lf, and was, therefore, comparatively richer in antitoxin (Table I). The amount of antitoxic nitrogen precipitated by 1.0 Lf was determined in optimal proportions by subtracting from the total precipitated nitrogen the value of 0.00046 mg. per Lf, the amount of toxin nitrogen associated with 1.0 Lf of toxin (13). Table I shows that, whereas recent pseudoglobulin preparations gave 0.0016 mg. of N per Lf, which agrees with Pappenheimer and Robinson's data (13), split antitoxin gave on the average only 0.00117 mg. There was thus a perceptible correlation between the smaller molecular weight of split antitoxin and the decrease in specifically precipitated antitoxin nitrogen. The values obtained remained fairly independent of the concentration of the solutions, which ranged from about 30 to 160 Lf per ml.

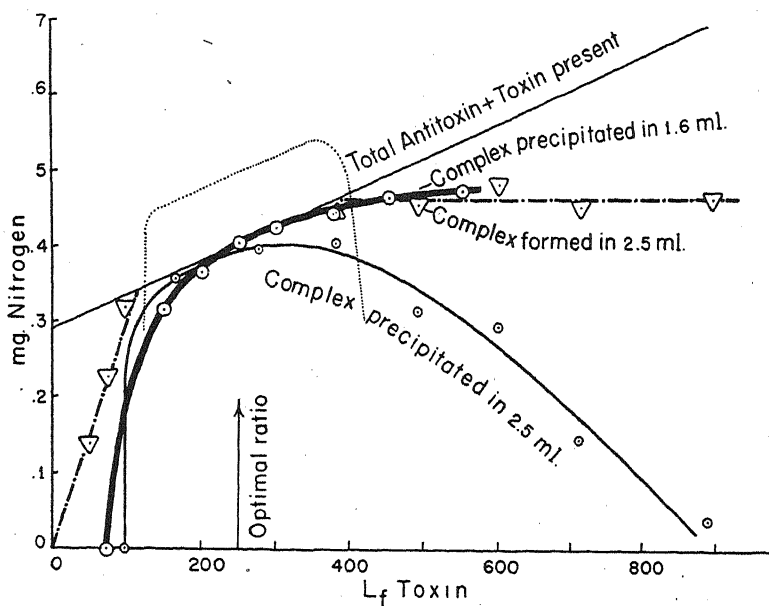


FIG. 1

Flocculation of 250 Lf of Split Antitoxin with Various Amounts of Toxin

Dotted line: Complex precipitated from fresh, untreated antitoxic pseudoglobulin, adapted from Pappenheimer and Robinson (13).

When attempts were made to determine the amount of nitrogen precipitated outside of the point of optimal proportions, concentration, among other possible factors, was found to influence the amount of uncombined reagent left in the supernatant (Table V) and also the amount of precipitate formed. The results from two detailed experiments have been summarized in Fig. 1.

TABLE V
Flocculation of 340 Lf of Split Antitoxin with 680 Lf of Toxin in Increasing Volumes of Solution .*

Total volume (ml.)	1.6	3.2	6.4	12.8
Toxin remaining in supernatant after first flocculation (Lf)	105	110	155	180

* Phosphate saline, pH 7.1.

The straight line was drawn on the basis of 0.00117 mg. of nitrogen per Lf of antitoxin and 0.00046 mg. of nitrogen per Lf of toxin. It gives the total amount of active nitrogen present. The two main curves show that the amount of nitrogen precipitated in optimal ratio was the same when the volume was 1.6 or 2.5 ml. In the region of toxin excess, however, much less complex was precipitated when the volume was 2.5 ml. In order to estimate the total amount of complex, soluble plus insoluble, formed in the latter region, the supernatant after removal of the precipitate was titrated. The Lf value thus obtained was translated into milligrams of nitrogen and subtracted from the value for total active nitrogen given by the straight line. In the region of antitoxin excess, where no precipitate appeared, the amount of soluble complex formed was estimated in a similar manner. The results indicate that with antitoxin in excess the amount of complex formed was fairly proportional to the amount of toxin added, whereas with toxin in excess the antitoxin reached a definite saturation point above which all of the excess toxin added remained free. In general, results obtained in the zone of toxin excess were not closely reproducible, and tubes from duplicate determinations sometimes yielded amounts of precipitate differing even to the naked eye.

DISCUSSION

Observing that less nitrogen was precipitated by toxin from enzyme-treated diphtheria antitoxin than from untreated antitoxin, Pappenheimer and Robinson (13) and Pope (14) first suggested that proteo-

lytic digestion caused a reduction in the size of the antitoxin molecules. This was confirmed by the sedimentation studies of Petermann and Pappenheimer (15, 16, 17) who showed that enzyme treatment of normal as well as of antitoxic pseudoglobulin resulted in the appearance of smaller protein molecules, roughly one half and one quarter of the original, together with dialyzable nitrogenous products. Peptic digestion at pH 4.2 was thus found by sedimentation measurements to reduce the molecular weight of diphtheria antitoxin from about 184,000 to 113,000 (15). On the other hand, Rothen (10) found a weight of about 90,000 for the pure antitoxin obtained by Northrop from a tryptic digest of the toxin-antitoxin complex. The results appear, in general, to be essentially the same regardless of the enzyme used, as long as digestion takes place at an appropriate pH.

In the absence of any evidence to suggest that changes of this type can be induced without the help of proteolytic enzymes, it may be assumed that the pseudoglobulin studied in the present instance underwent a process that was due to the presence of some native enzyme in the original serum. This view would be supported by Pope's observations on the role of fibrinolysin (14). That this spontaneous splitting process is common in old pseudoglobulin preparations is indicated by the fact that three other samples from eight to nine years old yielded to an equal or less extent, upon treatment with CuSO_4 , a fast flocculating fraction of lower protein:antitoxin ratio than the original.

The data reported here show that there had occurred, in diphtheria antitoxin lot No. 626A, a decrease in mean molecular weight from a probable 165,000 to about 130,000, or 20%, whereas the decrease in total protein nitrogen was only 9.0%. This indicated that some of the molecules present were derived from the splitting in two of original molecules. That this splitting resulted in the formation of unequal halves is suggested by the fact that the molecular weight (by osmotic pressure) of the resistant fraction was decidedly more than half that of intact molecules. From the sedimentation measurements, on the other hand, it may be estimated that about 30% of the material was present as approximate half-molecules ($S_{20}^0 = \text{ca. } 5.0 \times 10^{-13}$), while the rest was, on the average, of slightly decreased molecular weight. It should be noted that there were not enough intact molecules left to give, in the sedimentation diagram, a peak corresponding to a normal constant of about 7.0.

As regards the antitoxin molecules proper contained in the split

fraction, they must have represented $0.00117:0.0065 = 18\%$ of the total protein (Table I). It is probable that physically they did not differ much from the non-antitoxic molecules. This view is warranted by the homogeneity of the fraction during sedimentation, the fair proportionality in native and in split antitoxin between molecular weight and specifically precipitated nitrogen, and also by the approximate constancy of the protein:antitoxin ratio observed when fractional ammonium sulfate precipitation was attempted.

The toxin binding power of split antitoxin seems, from Fig. 1, to have been essentially similar to that of native antitoxic pseudoglobulin. The complex was soluble in the same zone of antibody excess as native antitoxin, but was much less soluble in antigen excess. The amount of precipitate formed in the latter zone appeared, as already mentioned, to depend primarily on total concentration. In optimal proportions, on the other hand, the amount of nitrogen precipitated was fairly constant and independent of concentration; furthermore, it may be added that the L_+/L_f ratio of split antitoxin was about the same as that of the original material when fresh (0.8).

These facts suggest that the point of so-called optimal proportions had the same significance with split antitoxin as with the native product. Since the amount of nitrogen associated with 1.0 Lf of native antitoxic pseudoglobulin (molecular weight 163,000) is about 0.0016 mg. (13), whereas with the present split antitoxin (molecular weight 100,000–115,000) it was about 0.00117 mg., the number of molecules associated with 1.0 Lf must have been about the same in both cases. Taking 74,000 for the molecular weight of toxin (15) one may calculate from Fig. 1 that the antitoxin:toxin molecular ratio was about 2 in optimal proportions, 4 in antitoxin excess, and 1 in toxin excess, approximately the ratios estimated by Pappenheimer, *et al.* (9) for native antitoxin. These similarities bear out the contention (15) that split antitoxin differs from native antitoxin primarily in the fact that each molecule loses about 40% of its weight without loss of antitoxic power and without significant changes in the configuration of the active part.

SUMMARY

A twelve-year-old sample of pooled diphtheria antitoxic pseudoglobulin was studied and found to have spontaneously undergone partial hydrolysis.

This material contained a fraction resistant to denaturation by acid and by metal salts, and characterized by a decreased protein:antitoxin ratio and a shortened flocculation time.

Osmotic pressure determinations and measurements of sedimentation rates indicated that this fraction represented about 30 per cent of the original and had a molecular weight of from 100,000 to 115,000, as opposed to 163,000 for fresh pseudoglobulin. The toxin binding power of this split antitoxin was quantitatively studied and found essentially similar to that of fresh antitoxin. The mean value of 0.00117 mg. was obtained for the amount of split antitoxin nitrogen associated with 1.0 Lf.

Evidence suggested that partial spontaneous hydrolysis is a common phenomenon in aged antitoxic pseudoglobulin.

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Effects of Thiamine and Riboflavin Deficient Diets on Rats Differing in Their Efficiency of Food Utilization¹

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INTRODUCTION

Two strains of rats differing significantly in their efficiencies of food utilization were first segregated by Morris, Palmer, and Kennedy (1933), and further developed by Palmer and Kennedy. These two strains of rats have been inbred for the past 15 years. This, together with careful selection, has made possible the development of two strains which, on the average, are uniformly and markedly different in growth rates and efficiencies of food utilization. Extensive study has revealed definite inherent physiological differences between the "high efficiency" strain and the "low efficiency" strain animals. In the present study the effects of deficiencies of thiamine and riboflavin on the content of these vitamins in body tissues were investigated.

The question of the interrelationship of thiamine and riboflavin presents an interesting but controversial picture. Sure and Ford (1942), while studying interrelationships of thiamine and riboflavin in the rat, found that thiamine deficiency caused a pronounced disturbance in riboflavin metabolism mainly because of poor absorption of the latter.

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However, in riboflavin deficiency there was no disturbance in thiamine metabolism. Ferrebee and Weissman (1943) were unable to demonstrate that thiamine deficiency has any effect upon riboflavin metabolism in the rat. Changes in riboflavin metabolism which occurred in the later stages of thiamine deficiency were not considered significant. In a later paper Sure (1944) found that if rats were maintained on sub-optimum levels of thiamine intake for a period of 6 months, large excesses of riboflavin were excreted in the urine. In a study of the interrelationship between thiamine and riboflavin Singher, *et al.* (1944) found that the concentration of riboflavin in the liver of the thiamine deficient rat was markedly increased over that of control animals. When control animals in both groups were restricted in their food intake to that of the deficient groups, no such increases were noted.

That there are strain differences in the thiamine requirements of rats was first observed by Nilson (1936). Later, Light and Cracas (1938) also reported that an inherent difference in thiamine requirements existed between different strains of rats. Engel (1943) has shown that the choline requirement of rats is determined by heredity. Two strains of rats were obtained, one having a choline requirement of at least twice that of the other group.

EXPERIMENTAL

Three groups of rats each composed of 20 animals, 10 of which were from the strain having a high efficiency of food utilization, and 10 from the strain having a low efficiency of food utilization, were fed the same B-free basal ration which had the following composition: purified casein, 20%; sucrose, 66%; salts, 4%; lard, 8%; cod liver oil, 2%. The ration was fed *ad libitum* to all groups and records kept of the weekly food consumption of each animal.

The daily vitamin B supplement in micrograms per rat was thiamine 80, riboflavin 160, pyridoxin 120, and calcium pantothenate 100. Choline chloride in the amount of 10 mg. per day per rat was also added. Aqueous solutions of these supplements were so prepared that the daily requirement of all vitamins was contained in 2 ml. The desired vitamin deficiency was produced simply by excluding that vitamin from the supplement.

When characteristic symptoms of each vitamin deficiency appeared, the rats were sacrificed and the liver and kidney tissue of each rat assayed for thiamine and riboflavin.

For the determination of thiamine in rat tissues the thiochrome method of Hennessy (1941) was used. Riboflavin assays were carried out using the microbiological method of Snell and Strong (1941).

RESULTS

The length of the feeding period for the group of normal control animals was arbitrarily selected as six weeks. During this period the average gain in weight of the animals of the high efficiency strain was 38% greater than that of the low efficiency strain animals while the average food intake of the high efficiency strain animals was only 5% greater.

Vitamin Composition of Thiamine Deficient Rats

When the thiamine-free ration was fed to the two strains of rats the time required for the low-efficiency strain animals to develop acute polyneuritis averaged 58 days, but only mild symptoms of thiamine deficiency were produced in the high efficiency strain rats even after 90 days of the same diet. The high efficiency rats did not show the typical spasms brought about by handling although the characteristic extension of the legs and peculiar spastic gait were observed. Thus, while symptoms of mild thiamine deficiency developed, no case of acute polyneuritis was observed in animals of the high efficiency strain.

Examination of Table I shows that the mean thiamine content of

TABLE I

Effect of Thiamine and Riboflavin Deficiency on the Mean Vitamin Content of Fresh Kidney and Liver Tissue of the Two Strains of Rats

Group	Strain	Organ	Thiamine		Riboflavin	
			µg./g.	S.D.	µg./g.	S.D.
Normal	H.E.	Kidney	7.03	0.80	35.66	2.87
Thiamine Deficient	H.E.	Kidney	5.43	0.62	37.36	2.10
Riboflavin Deficient	H.E.	Kidney	9.11	1.23	16.84	2.47
Normal	H.E.	Liver	9.93	1.17	24.61	1.63
Thiamine Deficient	H.E.	Liver	6.35	1.23	37.36	4.66
Riboflavin Deficient	H.E.	Liver	15.55	2.30	9.20	1.95
Normal	L.E.	Kidney	5.27	0.67	31.42	3.93
Thiamine Deficient	L.E.	Kidney	1.20	0.28	51.84	8.57
Riboflavin Deficient	L.E.	Kidney	7.56	1.69	18.55	2.12
Normal	L.E.	Liver	6.71	1.27	27.34	4.54
Thiamine Deficient	L.E.	Liver	1.24	0.34	48.61	7.10
Riboflavin Deficient	L.E.	Liver	16.29	3.05	10.41	1.38

H.E.—High Efficiency Strain.

L.E.—Low Efficiency Strain.

the liver and kidney of the low efficiency strain is considerably lower than the mean thiamine content of the same organs of the high efficiency strain. Thus, the thiamine concentration in the organs of the high efficiency strain rats is not low enough to cause symptoms of acute polyneuritis. No explanation can be offered for this difference in response to a thiamine-free ration except that the high efficiency strain undoubtedly has a much lower requirement for this vitamin.

The riboflavin concentrations in the livers and kidneys of the thiamine deficient rats of both strains are much greater than that found in normal control rats. These high concentrations of riboflavin in liver and kidney tissue of the thiamine deficient rats represent a very large apparent increase over that of the normal controls. The increase of riboflavin in the liver of thiamine deficient rats was first reported by Singher, *et al.* (1944) who found that restricting the food intakes of the control animals did not appreciably affect the riboflavin content of the liver.

Vitamin Composition of Riboflavin Deficient Rats

Striking differences between the two strains of animals were observed after only two weeks on the riboflavin-free ration. At this time the fur of the high efficiency animals became moist and matted with a definite though small loss of hair on the median line of the head, but the low efficiency strain animals appeared normal in every respect. At the end of the fourth week alopecia had progressed further in the high efficiency strain and declining weight was also noted. Symptoms of alopecia had been reached in the high efficiency animals and they were killed and analyzed. No alopecia was noted at this time in the low efficiency rats; however, four out of the ten rats declined slightly in weight. It was not until the thirteenth week that alopecia had progressed to the same point in the low efficiency rats as it had in the high efficiency animals at the sixth week. However; an additional symptom of riboflavin deficiency appeared in rats of the low efficiency strain at about the seventh week, and this was the development of corneal vascularization. The slow onset of corneal vascularization explains why it did not develop in the animals of the high efficiency strain.

The results of the vitamin analysis of the kidneys and livers of the riboflavin deficient rats are shown in Table I. It can be seen that even though it required twice as long to produce the same condition of

alopecia in the low efficiency strain as it did in the high efficiency strain rats, the riboflavin contents of the tissues of both strains are the same when the same stage of alopecia has developed. This indicates that the body concentration of riboflavin must be reduced to a certain level before severe alopecia will become apparent.

Again it can be seen from the table that the thiamine concentration in the livers and kidneys of riboflavin deficient rats is higher than that of the normal group. Singher, *et al.* (1944) report that the thiamine concentrations in the livers of riboflavin deficient rats were higher than the controls irrespective of whether or not the food intake of the controls was restricted to that of the riboflavin deficient animals.

DISCUSSION

The effect of a thiamine-free diet on animals of both strains indicates a much lower requirement for thiamine in animals of the high efficiency strain. The hypothesis of greater intestinal synthesis of thiamine in animals of the high efficiency strain is possible; however, the nature of the basal ration would seem to preclude extensive bacterial synthesis of this vitamin.

The very high concentrations of riboflavin in liver and kidney tissue of thiamine deficient rats are difficult to explain. The claim of Sure and Ford (1942) that in the thiamine deficient rat riboflavin is poorly absorbed is not substantiated by this work. In fact, the contrary would seem to be the case as is borne out in the work of Singher, *et al.* (1944).

The effect of the riboflavin free diet on both strains of rats seems to indicate that the riboflavin requirement of the high efficiency strain animals is at least double that of the low efficiency strain rats. This fact is supported by the result of preliminary experiments, not reported in this paper, whose purpose was to determine the optimum levels of each vitamin in the vitamin supplement necessary to insure ample protection from deficiency. In the first supplement prepared, riboflavin was fed at a level of 80 μ g. per day per rat to several rats of both strains. Within two weeks symptoms of early riboflavin deficiency appeared in the high efficiency strain animals, while the low efficiency strain animals remained normal. When the level of riboflavin was increased to 160 μ g. per day per rat the symptoms of riboflavin deficiency disappeared in the high efficiency strain. The high concen-

tration of thiamine in the liver and kidney of riboflavin deficient rats is similar to that found by Singher, *et al.* (1944) for liver tissue. The concentration of thiamine in the liver and kidney of riboflavin deficient rats is two to four times greater than that found in the normal control group.

SUMMARY

Two strains of highly inbred rats segregated and developed by genetic means on the basis of efficiency of food utilization have been found to differ in their response to a thiamine and riboflavin free diet.

Pronounced strain differences were found with respect to the thiamine requirement as evidenced by the fact that acute polyneuritis could be produced in the low efficiency strain animals in an average of 58 days while only mild deficiency symptoms were produced in animals of the high efficiency strain after 90 days on the same thiamine free diet.

The riboflavin requirement of the high efficiency strain animals was found to be approximately double that of the low efficiency strain rats.

A low requirement for thiamine and a high requirement for riboflavin appear to accompany high efficiency of food utilization.

Certain reciprocal relationships were found to exist between thiamine and riboflavin. Thus, thiamine deficiency results in an increase in riboflavin concentration and riboflavin deficiency results in an increase in thiamine concentration in the liver and kidney tissue of rats.

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Significance of Magneto-Chemistry in Studies of Trace Metals (I). Linkage of Nitric Oxide to Copper

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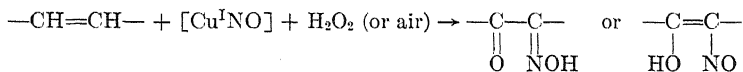
That metallic ions often are associated with vitamin and enzyme reactions is currently accepted. Our knowledge of the molecular mechanism of such reactions is very limited. An investigation of the problem is very difficult, because occasionally even infinitesimal amounts of a metal bring about extensive biological changes. However, it is evident that pronounced effects with trace elements are often found with enzymes or vitamins. Usually metallic combinations, as in hemin, appear to function as prosthetic groups in enzyme systems. It is presumed that the technique with tracer or labeled elements, particularly with radioactive metal combinations, will elucidate this rather obscure realm of science. In this study of metallic catalysts it is important to bear in mind that in certain enzyme systems the plain metallic ion or the atomic metal reacts as a prosthetic group and that the metal is unique for the specific enzyme.

For instance, in the case of polyphenol oxidases, according to Kubowitz (1), copper is linked directly to the enzymatic protein. No other metal can replace copper. This seems strange, since the catalytic action of the metal is usually explained by its change in valence or electronic transfer. There are many other metals in which a change of valence occurs under given conditions. Since copper cannot be replaced by any other transition metal, it is important to investigate the reason for this specificity and learn why copper is unique. No doubt there are other qualities which develop in the copper atom after the combination of the copper and the protein has taken place. Are these changes subatomic and do we have means to investigate and analyze them and study the function of copper and of other trace elements? In order to gain access to these subatomic realms we must first deter-

mine the types of bonding by which the metal is linked to the enzymatic system. From the type of bond which exists between the central metal atom and the atomic groupings, the magnetic properties of the central atom can be deduced, or *vice versa*. At the same time the characteristics and chemical structure of the attached organic groupings with which the trace element has formed a new complex are of importance. Since proteins are involved in enzymatic reactions, the problem of finding the details of the mechanism is difficult and complex. A simple chemical reaction must be found, in which copper reacts uniquely, or almost uniquely, and in which the attached atomic grouping is a simple inorganic or organic nitrogen compound. During such a reaction the characteristics of the simple metallic ion before and after binding, the nature of the added atoms, and the chemical and physical properties of the resulting molecule can be studied. It is decidedly important to find out the type of bonding that occurs between the metal and the attached groupings. For clarity, the names and symbols which are conventional in explaining the bondings, for instance between M and S , are as follows:

1. Electrovalence, $M^+ + S^- \rightarrow M^+[S^-]$
2. Co-valence, $M^+ + S^- \rightarrow M:S$
3. Donor-acceptor valence, $M: + S \rightarrow M:S$

In this paper the metal to be investigated is copper and the substance attached is NO. It will be seen later that in compounds all three kinds of bondings are to be found between certain metals and NO. In our example we have the simplest case of a copper to nitrogen attachment, which also takes place in the attachment of copper to the enzymatic protein. The new reaction, called B reaction for brevity, in its simplest form can be expressed by the following scheme.

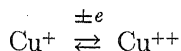


The writer has already published several articles on the B reaction (2), the mechanism of which remained rather obscure. A new simple procedure which makes the application of the B reaction very convenient has been found. If metallic copper is dissolved in an aqueous solution of $\text{NH}_2\text{OH} \cdot \text{HCl}$ in the presence of air, a colorless clear liquid, L , is obtained which contains a cuprous salt of nitrosyl ($\text{Cu}^{\text{I}}\text{NO}$). This combination is most reactive and can be used to add NOH to

ethylene linkages. If for instance benzene is shaken at room temperature with solution *L*, some of the benzene is immediately converted into *o*-nitrosophenol. Since this compound with cupric ions gives a deep red water-soluble complex salt, the colorless solution *L*, on being shaken with benzene, becomes pink if the necessary oxidation is brought about by air, or intensely red if dilute H_2O_2 has been added to *L*. That solution *L* does attack benzene (toluene, xylene, etc.) quickly at room temperature is astonishing, and a closer study of the mechanism of the reaction is of general importance for enzyme chemistry, especially when copper alone is involved.

Let us first analyze why copper ions should differ from other ions of the transition elements. It has been found that in the B reaction only mercurous ions and silver ions can replace copper, but they are much less effective, and nothing more will be said about these elements in this paper. We shall, however, analyze the properties of copper and of the attached NO in greater detail from the viewpoint of magneto-chemistry.

In regard to the magnetic properties of copper ions, it is known that cuprous ions (18 electrons) are diamagnetic. It has, however, been found that a feeble paramagnetism is present (3), a fact which seems to be of significance in the linkage of NO to cuprous ions. Cupric ions (17 electrons) possess the paramagnetism of about 1.73 Bohr magnetons. There is no other metallic ion in which the difference in magnetism of the two valence states of the simple (not complex) ions is so conspicuous as in the copper ions Cu^{I} and Cu^{II} . Cuprous ions are practically diamagnetic, while cupric ions are paramagnetic. Another difference between copper ions is that cuprous ions usually have a greater tendency to form an octahedral arrangement in space, while cupric ions possess a square or tetrahedral structure. For instance, the Cu^+ ion links 6 molecules of pyridine, while the Cu^{++} ion links only 4. In regard to the change in valence of copper ions,



it is significant that this change takes place easily in a weakly acid solution with an oxidation potential of a low voltage. The oxidation potential for the release of an electron from the cations of other transition elements is far too high to occur in acid solutions.

	Oxidation potential
$\text{Mn}^{++} \rightarrow \text{Mn}^{+++}$	1.5 volt
$\text{Ni}^{++} \rightarrow \text{Ni}^{+++}$	very high
$\text{Co}^{++} \rightarrow \text{Co}^{+++}$	1.8 volt
$\text{Fe}^{++} \rightarrow \text{Fe}^{+++}$	0.75 volt
$\text{Cu}^{+} \rightarrow \text{Cu}^{++}$	0.2 volt

However, the oxidation potential can be altered by the formation of complex coordinated ions. The addition of nitrogen compounds lowers the potential of copper ions and makes the valence shift still more easily in acid solutions.

Another peculiarity of copper ions which distinguishes them from ions of the other transition elements is the fact that attached atom groupings or negative ionic groups in the *second* sphere in the Werner complex influence the coordination number of the atoms or molecules linked in the first sphere.

From these few examples it can be seen that the chemical and physical characteristics of copper ions differ conspicuously from those of simple ions of other elements of the transition group. In this presentation we are naturally primarily interested in finding out why copper ions are almost unique in the B reaction, a specificity which is not often found in reactions *in vitro*. Reactions *in vivo* or reactions of biological origin are different. Since Kubowitz's discovery that polyphenol oxidase is a copper-protein complex, there have been published a number of papers showing that other enzyme systems which catalyze the oxidation of phenolic compounds belong to this group. To our knowledge, not much effort (if any) has been made to try to explain the remarkable specificity of copper in the polyphenol oxidase reaction or in any other biological reaction in which copper behaves differently from other metallic ions of the transition element group.

Such studies should be of great help in understanding the biological function of the trace elements, the importance of which becomes more and more recognized in plant and animal metabolism. Enzymes seem to be the most adequate compounds to study in this field. In this correlation, magneto-chemical studies should be of great help. Welo and the writer have found that compounds with an odd (non-polar) electronic number are good catalysts (4). It is to be assumed that the type of bond and the nature of the attached atomic groupings to the central metal atom determine in great part the characteristics of the complex formed, and thus of the enzyme system which possesses a metal

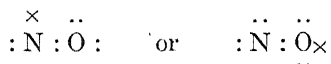
atom as a centrally located atom. From the type of bond between the central atom and the attached atoms or molecules, its magnetic property can be determined, or *vice versa*. In our experiments it is of great significance that the metallic ion which forms the center of the complex is weakly paramagnetic; the attached molecule, namely NO, possesses an uncompensated electron spin which makes it paramagnetic and also the resulting copper nitroso radical $[\text{Cu NO}]$ is paramagnetic.

In this paper we are concerned with nitric oxide, NO, a compound which has always been of great importance to chemists but only recently to biochemists. The discovery that N_2O_5 is a constituent of the ozone layer of the stratosphere (5) is of great significance. Very small amounts of NO are omnipresent in the atmosphere (0.01 to 2 p.p.m.). Only in recent years have the peculiarities of the NO molecule been fully understood. It is necessary to present a few data for our investigation: NO with its 11 electrons is a paramagnetic gas. It has a great affinity for metals and metallic ions; for instance, the following elements bind NO and form nitrosyl compounds.

Mn	Fe	Co	Ni	Cu	Zn
	Ru	Rh	Pd		
	Os			Cd	

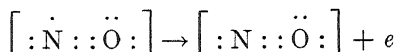
In the resulting nitrosyl or nitroso metal compounds the type of bonding is very different with the various metallic ions, and thus the chemical and physical behavior of the attached NO differs. The valence conditions of the metallic ions are of decisive importance. Both cuprous and cupric ions bind NO. The nature of the bond, however, is entirely different, and the mechanism of the B reaction is based on this difference in bonding of NO to copper ions and of the changes in valence: cuprous \rightleftharpoons cupric in an acid medium.

The electronic arrangement in NO is expressed by the following scheme.



Either the nitrogen or the oxygen atom can have the free spinning electron (x). NO has 3 electrons available for combination with other substances. Sometimes it uses all 3, sometimes only 2, and it can further react as a positive ion NO^+ which possesses only 10 electrons

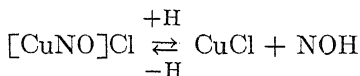
and thus is isosteric with CO. In this case the positive NO ion involves an oxidation:



In nitroprusside compounds $[\text{Fe}^{\text{II}}_{(\text{NC})_5}\text{NO}]$ the NO has been oxidized. The central iron atom is divalent. All the ferrous pentacyano compounds, as Welo and the writer found many years ago (4), are diamagnetic.

The NO in nitroprussides is more reactive, because it does not possess free spinning electrons. Some examples showing how NO reacts with different metals, using its 3 electrons in different ways, follow:

In the compound $\left[\begin{array}{c} \text{NO} \\ \text{Co}(\text{CO})_3 \end{array} \right]$ the NO donates 3 electrons to the cobalt atom (6) and, according to the Welo and Baudisch rule (4), the compound is diamagnetic. It has a krypton structure ($27 + 3 + 3 \times 2 = 36$). There are, however, metal nitroso complexes which are paramagnetic. If the electron shell of the reacting ions (with the minimum valence for the starting phase) has an even number of electrons, then the linkage of 1 NO molecule leads to a paramagnetic complex, the addition of 2 NO molecules to a diamagnetic complex. The cuprous ion complexes with NO have recently been studied magnetically by R. Asmussen (7). CuCl is diamagnetic and so is NOCl . When combined, a paramagnetic compound with monovalent copper is formed, $[\text{Cu}(\text{NO})]\text{Cl}_2$. Magnetic measurements prove that the NO has 1 unbound electron which represents the magnetic susceptibility of 1 Bohr magneton. NO is so loosely bound in the Asmussen complex that it can be driven out by CO_2 gas. Similar conditions were found when powdered copper metal was dissolved in an aqueous solution of $\text{NH}_2\text{OH} \cdot \text{HCl}$. The simplest way to carry out the B reaction successfully is described. By the following procedure a cuprous nitroso compound in which the nitroso group is loosely linked and is converted easily into nitrosyl, NOH, depending on a hydrating or dehydrating medium, is formed.

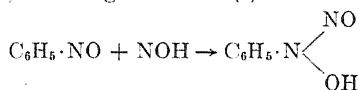


Procedure: 5 g. of $\text{NH}_2\text{OH} \cdot \text{HCl}$ are dissolved in 100 cc. of H_2O . To the solution, copper metal powder is added in excess (about 0.5 to 1 g.) and the mixture shaken on the machine for several hours. After the material has stood overnight in a closed

flask, the copper settles to the bottom and the supernatant liquid, *L*, is colorless and crystal-clear. The air above the liquid *L* becomes brown after the stopper is opened, because the NO above the liquid becomes oxidized to NO₂ and N₂O₃. The NH₂OH has thus been autoxidized to NO, and the liquid contains probably a copper salt of NO of the following simple structure Cu(NO)Cl, or the copper salt of the

Angeli acid $\text{NON} \begin{cases} \text{OH}, \\ \text{OH} \end{cases}$ or both. That the liquid *L* can be used as a source of

NOH is best demonstrated in the following experiment. To 1 part of *L* add an ethereal solution of nitroso benzene and put on the shaking machine. After a few hours shaking the liquid contains a silver-gray precipitate which can be separated easily by filtration. The gray precipitate is the copper salt of phenylnitrosohydroxylamine (cupferron-copper). The Angeli reaction (8)



takes place. Angeli obtained his reaction with nitrosobenzene and Angeli's salt

NONa
|
only in an alkaline medium, while in the new B reaction the liquid *L* is weakly
 NOONa
acid.

The liquid *L* can be used with success to demonstrate the B reaction. This is an excellent reaction to demonstrate how easily benzene is attacked at room temperature and two groups, NO and OH, are substituted in the ring.

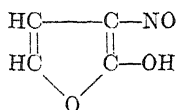
1 part of the clear liquid *L* is shaken with excess benzene or with pure ligroin which contains very small amounts of benzene. After the mixture is shaken for some time, a dilute H₂O₂ solution is added. The color of the aqueous layer almost immediately becomes intense red and the red deepens on further standing or shaking. *o*-Nitrosophenol copper which is soluble in water, with a deep red color, is formed. If the reaction should fail to occur immediately, the addition of small amounts of borax or guanidine will help to adjust the pH (4 to 6).

Dilute aqueous pyridine and CHCl₃ are added to the red mixture in order to produce free *o*-nitrosophenol. The red copper salt is taken up by chloroform when shaken. Wash the separated chloroform layer with ice water several times and add dilute KHSO₄ solution or dilute H₂SO₄. The red CHCl₃ becomes a beautiful green, and free *o*-nitrosophenol is formed. It can be converted to the red calcium salt by shaking with Ca(OH)₂ and kept in the refrigerator under pure ligroin for use in chemical analyses.

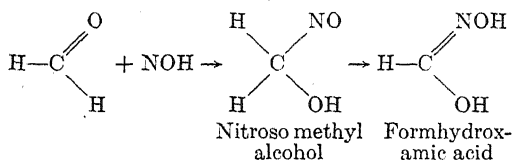
The copper nitroso compound present in the freshly prepared colorless liquid *L* can be extracted easily with organic solvents such as benzene, toluene, ethyl acetate, and others. In the absence of air this

extract is and remains colorless. It autoxidizes quickly in contact with air, and brown CuOH or other basic cuprous compounds are formed, while in the air above volatile NO can be recognized with filter paper strips dipped into Griess's reagent. On longer standing in contact with air all the copper precipitates out from the organic solvent in the form of a yellowish brown precipitate and the reactivity of the L solution is entirely lost.

The B reaction can be applied not only to aromatic hydrocarbons but many other ring compounds containing the ethylene grouping. The writer has found that furan is converted in small amounts to 3-nitroso-2-hydroxyfuran

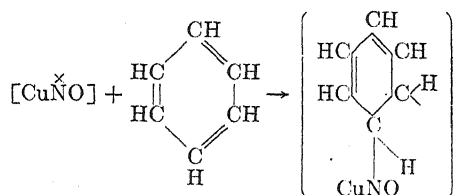


if furan is shaken at room temperature with solution L . The new nitroso compound shows the same basic color with metallic ions as *o*-nitrosophenol. It is of significance that, in any aromatic compound in which ethylene groupings are inserted, the $-\text{CH}=\text{CH}-$ groupings react spontaneously with the L solution, and, in the presence of air or H_2O_2 , *o*-nitrosohydroxy compounds are formed. The B reaction takes place only in an acid medium, and the best pH is usually from 2 to 6. The substitution of acid groups in the benzene nucleus has, however, a great influence on the shift of the pH to the acid side and on the stability of the metal complexes with the resulting *o*-nitrosophenols, making them more stable on the acid side. The nitrosyl reaction with Angeli salt or with Piloty's acid has always been carried out in a weakly alkaline medium. The writer, in conjunction with Coert (9), has first shown that the free NOH in neutral solution reacts usually as $\text{NO} + \text{H}$. For instance, *formaldehyde* and NOH give blue-green nitrosomethyl alcohol which is quickly rearranged to colorless formhydroxamic acid.



We have prepared about 70 new *o*-nitrosophenol compounds and tested them for complex formation with metallic ions. In another paper, Dr. G. Cronheim will describe these new compounds.

In regard to the mechanism of the B reaction, it is to be assumed that the benzene reacts with the $[\text{Cu}(\text{NO})]$ radical in the following manner:

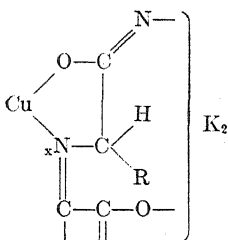


The labile intermediate compound becomes oxidized to *o*-nitrosophenol by air or oxygen or by H_2O_2 .

The ready change in the valence of copper ions in acid solutions is another feature of the mechanism of the B reaction. In addition to the radical reaction with radicals such as $[\text{Cu}(\text{NO})]$, NO, and H, it must be taken into consideration that a chain reaction takes place, and the radicals mentioned are members of the chain. Speculation concerning the mechanism of the actual chain reaction cannot be made at the present time, but further experimental work should reveal more facts and elucidate the mechanism of the B reaction still further.

The knowledge gained by studying the mechanism of the B reaction shall now be used in a discussion of the polyphenol oxidases in which copper is unique.

It is to be assumed that certain metals linked to polypeptides bring about a rearrangement of the molecular structure and, in general, activate the polypeptide molecules. For instance, in the formula below, the nitrogen atoms become magnets on account of uncompensated electrons and so acquire radical character. In such cases, we speak of resonance or electron isomerism. The enzyme action possibly occurs in a manner parallel to the formation of the stabilized free radical brought about by the donor-acceptor binding of copper ions. In this action we must seek the foremost value and importance of trace elements. If, for instance, copper reacts with a polypeptide, a compound of the following graphic structure is formed (10).



In this structure the nitrogen (N_x) linked to copper possesses an uncompensated electron or a micromagnetic field (Bohr magneton). Radicals in combination with metal ions or metal complex ions seem to be of fundamental importance in biological synthesis. It is important, furthermore, that a metal ion, like copper, which had already linked nitrogen-containing molecules and formed a Werner complex on account of such linkage receives selective affinity for other atoms, molecules, or groups. The copper atom, like other central metal atoms, has the tendency to complete or saturate its uncompensated coordination position by either donator or acceptor electrons.

SUMMARY

The mechanism of many chemical and biochemical reactions is governed by the nature or type of bondings of the atoms or atom groupings attached to the central metal atom. In the resulting compound the electronic structure of the metal might become altered considerably, and the attached atomic grouping may as well change chemical and spatial structure after having donated or accepted electrons. It is known by experience that the number and arrangement of electrons in a metal determine its characteristic mode of bonding other atoms, molecules, or groups. The writer has found a simple chemical reaction (B reaction) in which copper is almost unique in behaving as a central atom. The knowledge of the mechanism of the reaction should throw light on the uniqueness of copper in certain enzyme reactions. If NO is linked to cuprous ions, a water-soluble radical [CuNO] is formed in which the nitrogen atom possesses a great reactivity and unites with aromatic hydrocarbons at room temperature. NO linked to cuprous ions represents also a new source for nitrosyl, NOH, and the reaction mixture can be used for the synthesis of hydroxamic acids. The experiences obtained in studying

the mechanism of the B reaction are discussed in connection with enzymatic reactions.

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Formation and Reactions of Acetyl Phosphate in *Escherichia coli* *

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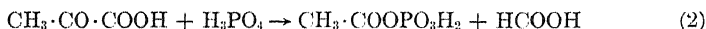
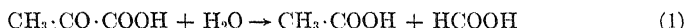
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INTRODUCTION

The formation of acetyl phosphate was first reported by Lipmann (9) during the oxidation of pyruvic acid by *Lactobacillus delbrückii*. The phosphate linkage in acetyl phosphate is of the energy-rich type (11) and is able to bring about the phosphorylation of adenylic acid.

A preliminary report (17) has shown that an acid-labile phosphate ester resembling acetyl phosphate is formed during the anaerobic dissimilation of pyruvic acid by cell-free extracts of *Escherichia coli*. The present communication presents further data on the accumulation of acetyl phosphate and its reactions in extracts of *E. coli*.

Kalnitsky and Werkman (3) showed that cell-free juices of *E. coli* were unable to attack pyruvic acid after short periods of dialysis but that the enzyme system was reactivated by the addition of inorganic phosphate. Previously Lipmann (11) had suggested that the so-called hydroclastic split of pyruvic acid (reaction 1) as thought to be caused by numerous species of bacteria might be in reality a "phosphoroclastic" split (reaction 2):



Recently Koepsall and Johnson (5) detected acetyl phosphate or a closely related labile phosphate during the anaerobic dissimilation of pyruvic acid by *Clostridium butylicum* (reaction 3):



Dissimilation of pyruvic acid by *Cl. butylicum* differs from that by *E. coli* in that the former forms CO_2 and H_2 rather than formic acid. The gaseous products apparently do not arise from formic acid since it is not attacked by the organism. In the case of the juice of *E. coli* Kalnitsky and Werkman (4) have shown by the use of heavy carbon that the formic acid does not arise from a reduction of CO_2 .

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The formation of acetyl phosphate during the anaerobic dissimilation of pyruvic acid by *Cl. butylicum* and *E. coli* offers a method by which the dissipation of energy during the reaction is avoided.

EXPERIMENTAL

E. coli (E 26) was grown on a medium containing 1% glucose, 0.4% yeast extract, 0.8% K_2HPO_4 , and 10% tap water. The cells were harvested by centrifugation after incubation for 24 hours at 30°C. The wet mass of cells was mixed with twice its weight of powdered glass and a little water or phosphate buffer and ground between concentric glass cones. The resulting paste was extracted with water or phosphate buffer in proportion of 1.5 ml. of liquid to 1 g. of wet cell paste. The glass was thrown out on an angle centrifuge and the extract further clarified by means of a Beams air-driven centrifuge. The clarified extract has already been shown to contain the enzymes necessary for the conversion of glucose to pyruvic acid (15, 16).

The extract could be preserved in a frozen state for several weeks although some decrease in activity occurred. Generally, the extract was used within a week. This enzyme preparation is similar but not identical to that obtained by Kalnitsky and Werkman (3, 4) from *E. coli* grown upon a non-carbohydrate medium. The preparation of the latter differed (a) in that succinic acid was formed from pyruvic acid, (b) with respect to the pH range, (c) the optimum phosphate concentration, and (d) in susceptibility to inhibitors. Both preparations, however, form acetyl phosphate during pyruvic acid dissimilation.

The experiments were conducted on a Barcroft-Warburg respirometer under anaerobic conditions at 30°C. unless otherwise indicated. Sodium pyruvate was prepared by neutralizing redistilled pyruvic acid; it was kept in a frozen state. Acetyl phosphate was prepared as the sodium salt from the synthetic di-silver salt.*

Pyruvic acid was determined by the colorimetric method of Straub (14). Inorganic phosphate was determined by the method of Fiske and Subbarow (1), and other phosphate compounds by methods which will be explained in connection with the experimental results.

Formation of Acetyl Phosphate

The experiments recorded in Table I illustrate the ability of the *E. coli* enzyme preparation to take up inorganic phosphate and form acetyl phosphate during the anaerobic fermentation of sodium pyruvate. The general method of Lipmann (10) for acid- and alkali-labile phosphate was followed in the determination of acetyl phosphate. The contents of the respirometer cup were rinsed into 5 ml. of ice-cold 2% CCl_3COOH , diluted to 10 ml., and quickly filtered. As soon as a small amount of filtrate had appeared, an aliquot was neutralized with ammonium acetate to approximately pH 8.0 using phenol red as the indicator. True inorganic phosphate was then precipitated with 3 cc. of a mixture of $CaCl_2$ and ethyl alcohol, centrifuged rapidly, and

* We wish to thank Dr. F. Lipmann for a generous sample of the synthetic salt.

TABLE I

Formation of Acetyl-PO₄ and ATP during Dissimilation of Pyruvate

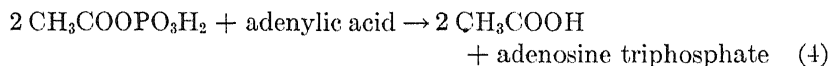
Expt. No.	Additions	Min.	Inorg. PO ₄ Disappeared	Acetyl-PO ₄ Formed	ATP Formed	Pyruvate Disappeared	Ratio PO ₄ Used/Pyruvate Disappeared
1	1.5 × 10 ⁻² mM Adenylic Acid (AA)	0	—	—	—	—	—
2	None	45	0.11	—	0.10	—	—
3	1.5 × 10 ⁻² mM AA	45	0.56	—	0.46	—	—
4	0.7 × 10 ⁻² mM AA	45	0.03	0.11	0.10	—	—
5	Pyruvate (6 × 10 ⁻² mM)	45	1.79	1.30	0.16	3.91	0.37
6	Pyruvate + 1.5 × 10 ⁻² mM AA	45	2.91	0.12	2.75	3.96	0.72
7	Pyruvate + 0.7 × 10 ⁻² mM AA	45	2.65	0.61	1.87	3.95	0.63

Each cup contained 0.8 ml. *E. coli* extract, 9 × 10⁻² mM NaHCO₃, 5 × 10⁻² mM PO₄ (pH 6.8) and indicated additions to 2.0 ml. Experiments conducted under an atmosphere of 90% N₂ and 10% CO₂ at 30.4°C.

Values in mM × 10⁻².

washed once with precipitation reagent. This procedure precipitates true inorganic phosphate, whereas acetyl phosphate is discarded with the centrifugate. Analysis of a similar aliquot by the usual method of Fiske and Subbarow (1) determines both acetyl phosphate and inorganic phosphate. The difference between the two methods is calculated as acetyl phosphate.

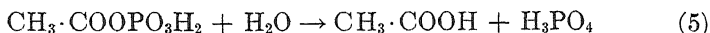
When no adenylic acid was present, acetyl phosphate was formed (Expt. 5). When 3 × 10⁻² mM adenylic acid was added to a 2 ml. volume, inorganic phosphate was converted to adenosine triphosphate, as indicated below:



Adenosine triphosphate was determined as the phosphate fraction hydrolyzable in seven minutes in 1 N HCl at 100°C. A lower concentration of adenylic acid (1.5 × 10⁻² mM) was insufficient to complete reaction 4 and some acetyl phosphate remained (Expt. 6).

Lipmann (10) found that approximately one mole of acetyl phosphate was formed for each mole of pyruvate oxidized by the *Lactobacillus* preparation. The last column of Table I indicates that the ratio of inorganic phosphate fixed as acetyl phosphate or adenosine triphosphate to sodium pyruvate utilized (PO₄/pyruvate) varies from 0.37 to 0.7 depending on the amount of adenylic acid present. Since acetyl phosphate is a labile substance, it seems possible that some of

the acetyl phosphate generated in the reaction may decompose if insufficient adenylic acid is present to accept the phosphate as shown in reaction 5. In this case the ratio $\text{PO}_4/\text{pyruvate}$ will be lowered:



Increasing the concentration of inorganic phosphate should slow the reaction since phosphate is one of the products. Table II shows that

TABLE II
Effect of Phosphate on the Accumulation of Acetyl Phosphate

Expt. No.	PO_4 Added $mM \times 10^{-2}$	Acetyl- PO_4 Formed	Pyruvate Disappeared	Ratio $\text{PO}_4/\text{Pyruvate}$
1	2.5	0.18	2.05	0.06
2	5.0	0.67	2.53	0.26
3	7.5	0.92	3.03	0.30
4	10.0	1.34	3.46	0.39
5	15.0	1.97	3.50	0.56
6	20.0	3.04	3.57	0.85

Each cup contained 0.8 ml. *E. coli* extract, 6.0×10^{-2} mM pyruvate, 9.0×10^{-2} mM NaHCO_3 , additions to 2.0 ml. Experiments conducted as in Table I. Time 45 minutes.

Values in $mM \times 10^{-2}$.

the concentration of phosphate does have a considerable effect in determining the accumulation of acetyl phosphate and subsequently the $\text{PO}_4/\text{pyruvate}$ ratio. The concentration of phosphate determines the rate of pyruvate utilization as well as the accumulation of acetyl phosphate. The optimum for pyruvate utilization is 0.05 M inorganic phosphate or above. This is considerably higher than reports by investigators working with similar systems. Kalnitsky and Werkman (3) found an optimum phosphate concentration of 0.015–0.02 M while the preparation of Koepsall and Johnson (5) from *Clostridium* showed a similar optimum.

With a phosphate concentration of 0.1 M or above the $\text{PO}_4/\text{pyruvate}$ ratio reached 0.8–0.85 but never attained unity. An analysis of the products of the fermentation (Table III) explained this apparent discrepancy. If phosphorylation is ignored, the main reaction carried out by the extract on pyruvate is represented by reaction 1 although another reaction (6) is involved to some extent:

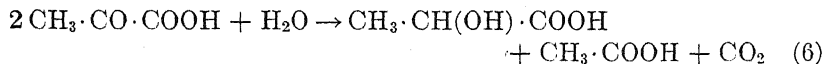


TABLE III

Products of Fermentation of Pyruvate by E. coli Extracts

Pyruvate fermented	1.49 mM
CO ₂ *	16.3 mM
Formic acid	60.6 mM
Acetic acid	84.6 mM
Lactic acid	13.5 mM
C-recovery	0.97%
O/R Index	0.93

* Products per 100 mM of pyruvate fermented.

Experimental cup contained 18 ml. of *E. coli* extract, 1.8 mM of pyruvate, 2 mM of phosphate buffer (pH 6.8) and water to a volume of 60 ml. The experiment was conducted under N₂ at 30.4°C.

Reaction 6 has been reported to take place in *Lactobacillus* (12), as well as in other bacteria (7) and in tissue. Analysis failed to disclose the presence of succinic acid or other 4-carbon dicarboxylic acids. The fermentation was carried out in 125 ml. respirometer flasks containing a total volume of 30 ml. of solution. The contents of two cups were combined and analyzed. CO₂ was determined manometrically; formic acid by oxidation with HgO (13). The remaining volatile acid was shown to be acetic acid by the partition method (13). Lactic acid was determined by the method of Friedemann and Graesser (2).

Since the enzyme extract showed little ability to break down formic acid to H₂ and CO₂ under the conditions of the experiment, most of the CO₂ must have arisen according to reaction 6. The lactic acid and CO₂ values of Table III indicate that 13.5% of the pyruvate fermented was reduced to lactate and was not available for the formation of acetyl phosphate *via* reaction 2. Accordingly the highest possible PO₄/pyruvate ratio would be 0.87. Since this value has been approached it is clear that one mole of acetyl phosphate does arise for each mole of pyruvate converted into acetic acid whether formed by the dismutation or phosphoroclastic reaction. The conversion of pyruvate to acetate and formate (reaction 1) is 4 to 5 times as rapid as the dismutation (reaction 6) under the experimental conditions. Apparently acetyl phosphate arises during the dismutation reaction as well as during the hydroclastic (or phosphoroclastic) reaction since the acetyl phosphate value approaches 0.85 with high concentrations of phosphate or with adenylic acid present as a phosphate acceptor. If acetyl phosphate were not formed during the dismutation reaction

the PO_4 /pyruvate ratio would be lower since some of the pyruvate is dissimilated by that reaction.

Effect of Various Factors upon the Accumulation of Acetyl Phosphate

The effect of time on the accumulation of acetyl phosphate is shown in Table IV. The ratio of PO_4 /pyruvate was fairly constant for the

TABLE IV

Accumulation of Acetyl Phosphate during Pyruvate Dissimilation by E. coli

Minutes	Inorg. PO_4 Disappeared	Acetyl- PO_4 Formed	Pyruvate Fermented	Acetyl- PO_4 / Pyruvate Fermented
15	1.03	1.10	2.37	.464
30	1.93	1.94	3.86	.502
45	2.48	2.54	5.43	.468
60	3.01	2.62	6.38	.411
75	2.82	2.66	7.32	.363

Each cup contained 0.8 ml. *E. coli* extract, 9.0×10^{-2} mM Na pyruvate, 12.5×10^{-2} mM PO_4 (pH 6.8) and a total volume of 2.0 ml. Experiment conducted under 100% N_2 at 30.4°C .

Values in mM $\times 10^{-2}$.

first 45 minutes but declined thereafter. A closer examination shows that acetyl phosphate increased until the 45 minute reading and remained fairly constant while the utilization of pyruvate continued thus causing a decrease in the PO_4 /pyruvate ratio.

The speed of pyruvate dissimilation and the accumulation of acetyl phosphate markedly was affected by pH as shown in Table V. The PO_4 /pyruvate ratio was higher at the lower pH values.

TABLE V

Effect of pH on the Accumulation of Acetyl Phosphate during Pyruvate Dissimilation by E. coli

pH	Acetyl- PO_4	Pyruvate Fermented	Ratio Acetyl- PO_4 /Pyruvate
5.6	0.62	0.72	0.86
6.2	1.03	1.47	0.70
6.8	1.86	4.80	0.39
7.4	1.56	5.22	0.30
8.0	1.27	5.26	0.24

Each cup contains 0.8 ml. *E. coli* extract, 6.0×10^{-2} mM Na pyruvate, 15.0×10^{-2} mM PO_4 of indicated pH and a total volume of 2.0 ml. Atmosphere 100% N_2 . Time 45 minutes.

Values in mM $\times 10^{-2}$.

Additional experiments showed that variations in the pyruvate concentration had little effect on the reaction. Variations in the atmosphere affected the activity of the preparations. Acetyl phosphate appeared and pyruvate was dissimilated rapidly under 90% N₂ and 10% CO₂, and also under a mixture of 90% H₂ and 10% CO₂ as well as under 100% N₂. The reaction was considerably slower under an atmosphere of 100% CO₂ and very slow in air.

Transfer of Phosphate from Acetyl Phosphate

Acetyl phosphate is of prime importance as a metabolic intermediate chiefly because it is a source of energy-rich phosphate (11). As such, acetyl phosphate should be able to transfer its phosphate group to a suitable acceptor such as glucose through mediation of the adenylic acid system. Lipmann (9) showed that adenosine triphosphate was formed during the oxidation of pyruvate by *Lactobacillus delbrückii* when adenylic acid was present. In other experiments Lipmann (8) showed that a similar preparation can transfer phosphate from synthetic acetyl phosphate to added adenylic acid. Koepsall, Johnson, and Lipmann (6) report a transphosphorylation of glucose from acetyl phosphate by means of an enzyme preparation of *Clostridium butylicum*.

An experiment (Table VI) was designed to determine whether phos-

TABLE VI

Transfer of Phosphate from Naturally formed Acetyl Phosphate to Adenylic Acid and Glucose

Expt. No.	Additions in Main Chamber	Contents 2nd side arm added at 45 min.	Time Min.	Inorg. PO ₄ Disappeared	Acetyl-PO ₄ Formed	ATP-PO ₄ Formed	Pyruvate Utilized
1	None	—	45	2.48	2.59	0.65	4.52
2	None	0.3 ml. H ₂ O	60	3.48	3.28	0.69	4.91
3	8 × 10 ⁻² mM NaF	0.3 ml. H ₂ O	60	4.20	3.37	0.78	4.63
4	None	.01 mM Adenylic acid	60	3.69	1.15	2.45	4.84
5	8 × 10 ⁻² mM NaF	.01 mM Adenylic acid	60	4.02	1.27	2.26	4.63
6	None	.04 mM Glucose	60	5.10	1.15	1.34	4.86
7	8 × 10 ⁻² mM NaF	.04 mM Glucose	60	5.82	1.66	1.09	4.84

All cups contain 0.8 ml. *E. coli* extract, 9.0 × 10⁻² mM NaHCO₃, 12.5 × 10⁻² mM PO₄ (pH 6.8), 6.0 × 10⁻² mM pyruvate, and additions to 2.0 ml. Atmosphere 90% N₂ and 10% CO₂.

Values in mM × 10⁻².

phate is transferred from acetyl phosphate to adenylic acid and glucose by the *E. coli* preparation. The experiments were carried out in respirometer cups with two side arms. After an equilibration period pyruvate was tipped into the extract-buffer solution. After an incubation of 45 minutes during which acetyl phosphate was generated from the pyruvate, the contents of the second side arm, adenylic acid, glucose, or water, were tipped in and the reaction allowed to proceed for an additional 15 minutes. An aliquot taken at the end of the first 45 minute period showed $2.59 \times 10^{-2} mM$ acetyl phosphate- PO_4 and $0.65 \times 10^{-2} mM$ of adenosine-triphosphate- PO_4 . The additional 15 minute period caused an increase in acetyl phosphate- PO_4 to $3.28 \times 10^{-2} mM$ when water was added. However, when adenylic acid was added to the cup after 45 minutes (Expt. 4), the acetyl phosphate value decreased to $1.15 \times 10^{-2} mM$ and the adenosine triphosphate value was correspondingly increased. Addition of glucose also caused approximately two-thirds of the acetyl phosphate to disappear although the increase in adenosine triphosphate was small, indicating that the phosphate compound formed in the reaction was not easily hydrolyzable. It is probable that the adenylic acid system catalyzes the transfer from acetyl phosphate to glucose. Sodium fluoride had little effect on the formation of acetyl phosphate or upon the transfer of phosphate from acetyl phosphate to adenylic acid or glucose.

To further determine the transphosphorylative powers of the *E. coli* extract, an experiment was carried out along similar lines with synthetic acetyl phosphate substituted for the naturally formed product as the phosphate donator. Since the experiment with glucose was to be conducted over a longer period of time, monoiodoacetic acid was added to stop the dissimilation of glucose which occurs fairly rapidly in *E. coli* extract. Phosphate was transferred readily from the synthetic acetyl phosphate to adenylic acid even in the presence of monoiodoacetic acid (Table VII). Approximately $3 \times 10^{-2} mM$ of acetyl-phosphate-P were transferred in 30 minutes. Glucose also accepts phosphate but to a somewhat less extent.

Koepsall, Johnson, and Lipmann (6) report that H_2 is inhibitory to the reaction in which pyruvate is broken down to acetyl phosphate, CO_2 , and H_2 by *Clostridium* and suggest that the reaction may be reversible.

Some evidence was obtained during these experiments that the phosphoroclastic reaction is reversible and further work is in progress.

TABLE VII

Transfer of Phosphate from Synthetic Acetyl Phosphate to Adenylic Acid and Glucose

Expt. No.	Additions	Change in Inorg. PO ₄	Change in Acetyl-phosphate-PO ₄	Change in ATP-PO ₄
1	None	1.0	-1.13	0.59
2	1.0×10^{-2} <i>mM</i> Adenylic acid (AA)	1.45	-3.93	2.77
3	1×10^{-2} <i>mM</i> AA, 2×10^{-2} <i>mM</i> Iodoacetic acid	1.55	-3.48	2.24
4	4×10^{-2} <i>mM</i> Glucose, 2×10^{-2} <i>mM</i> Iodoacetic acid	+0.55	-2.47	0.98

Each cup (unless otherwise indicated) contains 0.8 ml. *E. coli* extract, 7.5×10^{-2} *mM* PO₄ (pH 6.8), acetyl phosphate (4.0×10^{-2} *mM* labile PO₄) and additions to 2.0 ml. Atmosphere 100% N₂. Time 30 minutes.

Values in *mM* $\times 10^{-2}$.

SUMMARY

1. A labile phosphate compound resembling acetyl phosphate accumulates during the anaerobic conversion of pyruvic acid to acetic acid and formic acid by a cell-free preparation of *Escherichia coli*.

2. The accumulation of acetyl phosphate is aided by an increasing inorganic phosphate concentration.

3. Approximately one mole of acetyl phosphate arises for each mole of pyruvic acid converted to a two carbon compound.

4. The cell-free preparation is able to transfer phosphate from acetyl phosphate to added adenylic acid or glucose.

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Fixation of Carbon Dioxide in Lactic Acid by *Clostridium Butylicum* *

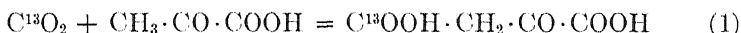
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INTRODUCTION

The primary reaction in the fixation of carbon dioxide by heterotrophic bacteria is generally considered to be a C_3 and C_1 addition with formation of a dicarboxylic acid (1).



It has been assumed that other compounds which have been found to contain fixed carbon, *e.g.*, propionic, acetic, and lactic acids, may be derived from dicarboxylic acids. This origin of the compounds is not definitely established, however. Slade, *et al.* (2) have reported the formation of lactic acid with fixed carbon in the carboxyl group by *Clostridium welchii* and by *Clostridium acetobutylicum*. Since these bacteria have not been reported to form dicarboxylic acids, it is evident that the proposed mechanism for fixation must be considered with reservation for these bacteria. It is possible that the lactic acid is formed by C_2 - and C_1 - addition. On the other hand, the dicarboxylic acid may be an intermediate which does not accumulate in measurable quantities.

The formation of lactic acid by *Clostridium acetobutylicum* had not been reported in the literature prior to the results of Slade, *et al.* For this reason there has been some doubt as to the reliability of the evidence that the butyl alcohol bacteria can fix carbon dioxide.

The object of the present investigation has been to obtain additional information concerning the fixation of carbon dioxide in lactic

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acid, and to determine whether the butyl alcohol bacteria fix carbon dioxide. The organism chosen for study was *Clostridium butylicum*. A large amount of lactic acid is formed by this organism from glucose when the pH is maintained approximately neutral with sodium bicarbonate (3). This procedure assured a substantial formation of lactic acid and provided enough material for a reliable degradation.

The results established conclusively that the butyl alcohol bacteria fix carbon dioxide in the carboxyl group of lactic acid. This fact raises the question whether the dicarboxylic acids play a heretofore unexpected intermediary part in the metabolism of these bacteria or do these bacteria have a mechanism of fixation of carbon dioxide different from that of reaction (1).

METHODS

The principal experiment was conducted as described by Osburn, *et al.* (3) except that sodium bicarbonate contained heavy carbon. The medium, 70 ml. in a 125 ml. Erlenmeyer flask, contained 2.00 g. of glucose, 0.7 g. of Bacto peptone, 0.2 g. Bacto yeast extract and 0.1 g. of K_2HPO_4 . The glucose was sterilized in distilled water and added to a sterile solution of the other constituents. The flask was fitted with a gas inlet tube and an outlet tube to which was attached a mercury trap followed by an alkali bead tower in which the carbon dioxide was collected. The medium was made anaerobic by sweeping out the air with nitrogen and was then inoculated with 0.5 ml. of a 20-day corn mash culture of *Cl. butylicum*, 2D. Incubation was at 37°C.

When growth became evident at 19 hours, 10 ml. of sterile 0.8 M $NaHC^{13}O_3$ was added. At 41 hours the fermentation was acid to bromothymol blue and an additional 15 ml. of the bicarbonate were added. This was somewhat too large an amount of bicarbonate and stopped the growth. At 68 hours the fermentation was reinoculated, and at 87 hours it was again vigorous and continued so until 135 hours when gas evolution slowed down and stopped at 144 hours. The apparatus was then flushed out with nitrogen to carry the carbon dioxide into the bead tower. The medium was found alkaline to bromothymol blue, was negative qualitatively for sugar and was positive for pyruvate with the sodium nitroprusside test.

The products of the fermentation were fractionated according to the customary procedure of the laboratory.

Residual $NaHC^{13}O_3$ was removed from the medium by acidifying, aerating and boiling the solution. The carbon dioxide was collected in the same alkali as used for the fermentation. The total carbon dioxide in the alkali was determined as a measure of the sum of the added $NaHC^{13}O_3$ and the carbon dioxide produced by the sugar fermentation.

The neutral volatile products were removed by alkaline distillation, oxidized with dichromate and determined according to Stahly, *et al.* (4). The butyl and ethyl alcohols thus were obtained as the corresponding acids and the isopropyl alcohol as acetone. The acetone was separated from the acids by alkaline distillation.

The volatile acids were obtained by a twelve-volume steam distillation of the acidified residue of the alcohol distillation. Since pyruvic and lactic acids are some-

what volatile, the distillate was neutralized, concentrated, again acidified, and then steam distilled. The formic acid was determined and removed from this distillate by oxidation with mercuric oxide (Osburn, *et al.* (5)), and C^{13} in the resulting carbon dioxide was determined as a measure of C^{13} in the formic acid.

The residue from the formic acid oxidation was filtered, neutralized, concentrated, and then acidified and steam distilled. The volatile acids in the distillate were free of formic acid and were determined according to Osburn, *et al.* (6).

The residues from the volatile acid distillation were extracted continuously with ethyl ether for 24 hours after adding sodium bisulfite to hold back the pyruvic acid. The extract was negative qualitatively for pyruvic acid and there was no precipitate with silver nitrate. Therefore no dicarboxylic acids such as succinic, malic, fumaric, etc. were present. By titration there were 6.12 molar equivalents of acid present. By the Friedeman and Kendall oxidation (7, 8), 6.15 *mM* of carbon dioxide and 6.00 *mM* of acetaldehyde were obtained. The non-volatile acid is thus shown to be lactic acid. The C^{13} was determined on the acetaldehyde which represents the α and β carbons and on the carbon dioxide, the carboxyl carbon.

The residue of the lactic acid extraction was boiled to remove the excess sulfite and was then extracted with ethyl ether for 24 hours to remove the pyruvic acid. By titration 0.16 m. eq. of acid were found. The extract was oxidized at room temperature with ceric sulfate (8). The resulting carbon dioxide was removed by aeration and 0.10 *mM* of carbon dioxide were obtained which was considered the carboxyl carbon of pyruvic acid.

The residue of the pyruvic acid extraction was oxidized with persulfate (9) and the C^{13} was determined in the resulting carbon dioxide. This measurement serves to detect fixed C^{13} in any unfractionated products.

Total carbon was determined on each fraction (16), *e.g.*, on alcohols, volatile acids, etc., and in each case the total carbon was found equivalent to the carbon calculated from the quantitative determination of the products. It is thus clear that the fractionation was adequate. The carbon dioxide from these total carbon determinations was used for determination of the C^{13} on the mass spectrometer.

A number of fermentations were set up to determine whether dicarboxylic acids such as malic, fumaric and succinic acids are fermented by *Cl. butylicum*. The dicarboxylic acids were added at the time of the first addition of bicarbonate or at the time of inoculation when no bicarbonate was to be added. The initial addition of bicarbonate was made as soon as active growth was observed. Subsequent additions were made when the reaction reached about pH 6.6 as judged from the color (green) of added bromothymol blue. The limit of tolerance to bicarbonate is close to the amounts added and in some instances growth was retarded by the final addition and the fermentation of the sugar was not complete. At the conclusion of incubation an aliquot of the medium was acidified and extracted continuously with ethyl ether for three days. The extract was steam

distilled to remove the volatile compounds and then the dicarboxylic acids were determined on the residue of distillation by precipitation as the silver salt. The amount of dicarboxylic acid was calculated on the basis of the particular dicarboxylic acid which was added. A small error is no doubt introduced by this assumption since there is partial conversion of fumarate to malate during the fermentation. However, for the present purpose the accuracy was sufficient.

One experiment was set up with a cell-free juice prepared from *Cl. butylicum*. This enzyme preparation was tested since inactivity with whole cells might be caused by impermeability to the test material. The enzyme was extracted with phosphate buffer (pH 6.6) from bacteria ground with powdered glass (22). The bacteria were harvested with a Sharples centrifuge from a 12 hour growth in a medium containing glucose 1.0%, Bacto yeast extract 0.2%, Bacto peptone 1.0%, and K_2HPO_4 0.1%. Activity of the enzyme was measured on a Warburg respirometer.

DISCUSSION

The results on the fixation of inorganic carbon from $NaHC^{13}O_3$ are shown in Table I. Of the various products only formic, lactic and

TABLE I

*Distribution of C^{13} of $NaHC^{13}O_3$ among Products of Fermentation by *Cl. butylicum* (2D)*

2.00 g. of glucose fermented; 87.7% of carbon of glucose recovered in products

	<i>mM</i>	Products per 100 <i>mM</i> substrate fermented <i>mM</i>	C^{13} in excess of normal per cent	C^{13} in excess of normal <i>mM</i>	Recovery of C^{13} of added $NaHC^{13}O_3$ per cent	Distribu- tion of fixed C^{13} per cent
Isopropyl alc.	0.05	0.5	0.01			
Butyl alc.	0.42	3.9	0.01			
Ethyl alc.	0.19	1.7				
Acetic acid	1.62	14.6	0.01			
Butyric acid	5.20	46.8				
Formic acid	1.06	9.5	1.60	0.017	2	13
Final $NaHC^{13}O_3 + CO_2$	30.6		2.39	0.732	79	
CO_2 (30.61-18.18)	12.43	111.8				
Lactic acid	6.15	55.4				
—COOH	6.15		1.89	0.116	13	87
$CH_3 \cdot CH(OH) -$	6.00		0.02			
Pyruvic—COOH	0.04	0.4	0.59	0.001	0.1	0.4
Residue Et ₂ O Ext.	42.7		0.02			
			Totals	0.866	94	
Original $NaHC^{13}O_3$	18.18		5.08	.924		

pyruvic acids contained a significant concentration of C^{13} in excess of the 1.09 per cent in normal carbon, *cf.* Column 4. Since the bicarbonate was the only source of carbon which contained C^{13} in a concentration greater than 1.09 per cent it is apparent that carbon from this source was present in the formic, lactic and pyruvic acids. The *mM* of C^{13} in excess of the normal carbon complement is shown in Column 5 and has been calculated from the data of Columns 2 and 3. From these figures the recovery of the C^{13} of the added $NaHC^{13}O_3$ was calculated. Ninety-four per cent of the added C^{13} is accounted for in the formic, lactic and pyruvic acids and the residual inorganic carbon. The greater part of the fixed carbon (87%) is present in the carboxyl group of the lactic acid. Obviously fixation of CO_2 in lactic acid is a major reaction in this fermentation. The concentration of C^{13} in the formic acid likewise was high but since the yield of this acid was lower than the lactic acid the total quantity of C^{13} fixed in formic is not as great. The determined per cent of C^{13} in the carboxyl group of pyruvic acid was lower than that for lactic acid (Column 4). However, there is some reason to question the reliability of this determination. The amount of pyruvic acid was small and therefore there may have been considerable contamination from extraneous carbon. It is probable that the true value for the C^{13} in the carboxyl of the pyruvic acid is about the same as in the lactic acid. As judged from the sodium nitroprusside reaction a large part of the pyruvic acid was decomposed during the fractionation procedure. This fact probably accounts for the somewhat low carbon recovery, which was 87.7% of the fermented glucose. On the basis of 100 *mM* of glucose Osburn, *et al.* report yields of pyruvic acid varying from 3.0 to 12.0 *mM*. Their lactic acid values were from 43 to 60 *mM* and the other products were in comparable amounts with the present experiment.

The fixation of CO_2 in the formic acid may be accounted for since the bacteria probably contained hydrogenlyase. There are a number of facts to consider, however, and the complete mechanism of the formation of formic acid is still an unsolved problem. Koepsell, *et al.* (11) have shown that an enzyme can be obtained from *Cl. butylicum* that breaks down pyruvate to acetic acid, CO_2 and H_2 ; and formate is apparently not an intermediate. The obvious conclusion from these observations would be that formate is not a primary product of pyruvate breakdown and that it is formed by a secondary reaction through reduction of CO_2 . If the formic acid was produced exclusively by

hydrogenlyase, it is to be expected that it would have a C^{13} content equivalent to the residual $C^{13}O_2$ and $NaHC^{13}O_3$. Actually the value is considerably less, 1.60 for formate as compared to 2.39% excess C^{13} for the residual $C^{13}O_2$ and $NaHC^{13}O_3$. This fact indicates that there may be some other mechanism for formation of formic acid which does not involve CO_2 reduction. Kalnitsky, *et al.* (12) have shown with a cell-free juice from *E. coli* that formate is formed from pyruvate in the presence of $C^{13}O_2$ with no substantial fixation of $C^{13}O_2$ in the formate. Formate is in this case formed without CO_2 reduction. The reaction requires phosphate so it is not a simple hydroclastic reaction. A similar reaction may have occurred in the fermentation of the present investigation. In this case labeled formate would be formed since the pyruvate is shown to contain fixed carbon. This reaction thus can account for the formation of both labeled and non-labeled formate without recourse to hydrogenlyase.

On the other hand it is conceivable that the CO_2 is fixed in formate by hydrogenlyase and that the labeled formate by interaction with a 2-carbon compound yields labeled pyruvate and lactate. Utter and Werkman (20) have shown the split of pyruvic acid into formic and acetic acids by *Escherichia coli* to be phosphoroclastic with acetyl phosphate and formic acid resulting. Acetic acid is then formed by the dephosphorylation of the acetyl phosphate. Reversibility of this reaction has been shown by Utter, Lipmann, and Werkman (21) using heavy carbon formic acid. Since CO_2 is normally in equilibrium with formic acid and the latter in equilibrium with pyruvic acid, CO_2 is fixed in the carboxyl group of pyruvic acid.

There was no indication of the formation of dicarboxylic acids in this fermentation and in the extensive literature on the butyl alcohol fermentation the authors have not found mention of the detection of dicarboxylic acids. There is thus no indication of 3- and 1-carbon addition, yet there is a large fixation of CO_2 in lactate. It is necessary to assume that the dicarboxylic acids are broken down as rapidly as they are formed, if it is concluded that the primary fixation of CO_2 is in dicarboxylic acids.

An attempt was therefore made to investigate this possibility. Dicarboxylic acids were added to fermentations and their rate of destruction was measured, the assumption being that they would be rapidly broken down if they are intermediates in the fixation of CO_2 in lactate. The results are summarized in Table II. Succinate, fumarate, and

TABLE II
Dissimilation of Dicarboxylic Acids by Cl. butylicum

No.	Ml. of 8-M NaHCO ₃ and hour of addition	Acid added mM	Original Volume ml.	Incubation hrs.	Starch or Glucose	Results of Fermentation				
						pH	Succinic acid-Pyruvic + CO ₂ mM	m.eq. of acid	Malate mM	Fumarate mM
Corn Mash ¹										
22	None	Succinic 3.0	100	72	—	Acid	3.1			
29	None	Succinic 3.0	100	192	—	Acid		7.0		
27	None	Fumarate 3.0	100	192	—	Acid		3.9		1.6
26	20 at 9, 10 at 80	None	60	154	+	Alk.		0.9		
28	20 at 9, 10 at 80	Fumarate 3.0	50	154	+	Alk.		3.5		0.4
30	20 at 9, 10 at 80	Succinic 3.0	50	154	+	Alk.	2.9	6.8		
32	20 at 11, 10 at 80	None	60	144	—	Acid		0.9		0.3
Glucose ²										
34	20 at 23, 5 at 94	None	65	110	—	Alk.	+			7.7
35	10 at 12, 10 at 24	None	65	75	—	Neut.	—			8.1
37	10 at 14, 10 at 72 ³ , 10 at 96	Fumarate 2.5	65	144	+	Alk.		8.05		1.37
38	10 at 25, 5 at 60, 10 at 96	Fumarate 5.0	65	120	+	Alk.				3.94
39	10 at 25, 10 at 60, 10 at 96	Malate 5.0	65	110	+	Alk.	+	11.6	4.2	1.2
40	10 at 25, 10 at 60 ³ , 10 at 84	Malate 2.5	65	110	+	Alk.	+	9.7	2.0	3.5
41	10 at 19, 5 at 42, 5 at 51	Malate 1.25	65	140	—	Neut.	+	12.2	1.1	6.2
		Fumarate 1.25								

¹ 3.75 g. corn meal, 0.5 g. Bacto Yeast Extract, 125 ml. Erlenmeyer flask, cotton plug, 37°C. Acid adjusted to pH 6.4 with NaOH solution except No. 22, adjustment was with K₂HPO₄.

² 2.0 g. glucose, 0.7 g. Bacto peptone, 0.2 g. Bacto Yeast Extract, 0.1 g. K₂HPO₄, 125 ml. Erlenmeyer flask, anaerobic with N₂, 37°C. Acids adjusted to pH 6.8 with NaOH solution.

³ Acid (yellow) to bromothymolblue at time of NaHCO₃ addition.

malate were added to corn mash and glucose fermentations. A rather wide variety of conditions was tested including those in which formation of lactate is large. Succinate remained unchanged throughout the fermentation. There was some destruction of malate, and fumarate was broken down more rapidly, however, the fermentation of fumarate was by no means complete or rapid, the greatest conversion being 1.9 *mM* during the course of a fermentation. There was no indication that the fumarate was converted to lactate. The milliequivalent of nonvolatile acid or lactic acid was not significantly increased above that of comparable fermentations to which no dicarboxylic acid was added. The results of this procedure were, therefore, not indicative of a fixation of CO_2 in dicarboxylic acids and a subsequent conversion to lactic acid.

It is of interest that under no circumstance was there a formation of lactate from corn mash that approached the large quantity that is formed from glucose. Repeated attempts to obtain lactate from corn mash by controlled addition of NaHCO_3 have failed. From glucose, lactate is readily obtained in good yield providing the bicarbonate is not added so rapidly that inhibition of the fermentation results. The reason for this difference in formation of lactate from corn mash and glucose is not evident.

The inactivity of the butyl alcohol bacteria on the dicarboxylic acids, when tested with whole cells, might result because of impermeability of the cells to the acid. In order to avoid this complication a cell-free enzyme preparation was tested and the results are shown in Table III. It was important to determine whether the bacteria contain the enzyme which decarboxylates oxalacetate to pyruvate, since this enzyme very likely catalyzes the primary fixation reaction in the formation of dicarboxylic acids (Krampitz and Werkman, 13, 15). The activity on succinate and malate was practically zero, on fumarate and lactate there was an initial activity which was lost very soon. The preparation was very active, however, on pyruvate and oxalacetate. The activity on pyruvate was almost as large as that on oxalacetate, which suggested that the evolution of CO_2 from oxalacetate might result largely from the fermentation of pyruvate formed from the spontaneous decomposition of the oxalacetate. Analysis of the residual oxalacetate by the aniline citrate method (16) showed that there were 922 $\mu\text{l.}$ of oxalacetate remaining at the end of one hour in the presence of the enzyme, whereas in the control to which no enzyme was added

1351 μ l. remained. This difference is not very impressive and is by no means comparable to the results obtained by Krampitz and Werkman (15). Oxalacetate is very labile and it is possible that the difference is caused entirely by nonenzymic changes (17). This change is not only a simple decarboxylation because the sum of the CO_2 liberated with acid and aniline is always less at the end of an hour's incubation than on a similar determination made at the beginning of the experiment. In the presence of the enzyme some oxalacetate may have been consumed by serving as a hydrogen acceptor thus being reduced to malate.

TABLE III

Activity of Cell-free Juice on Different Acids

<i>Minutes</i>	None <i>μl.</i>	Succi- nate <i>μl.</i>	Mal- ate <i>μl.</i>	Fuma- rate <i>μl.</i>	Lac- tate <i>μl.</i>	Pyru- vate <i>μl.</i>	Oxal- acetate <i>μl.</i>	Oxal- acetate (no en- zyme) <i>μl.</i>
6		3	11	136	—	—	244	
15		—	—	—	250	342	203	
25		4	9	58	23	297	363	
35		—	—	3	6	273	315	
50		—	—	3	4	251	345	
60	12	3	6	3	3	344	360	18
0-60	12	10	26	203	286	1507	1830	18
CO_2 with acid	0	0	1	23	26	153	630	512
Aniline	—	—	—	—	—	—	922	1351
Totals	12	10	27	226	312	1660	3382	1899

Calculated as equimolar mixture of H_2 and CO_2 except in case of liberation of gas by addition of acid and in case of oxalacetate control.

Reaction mixture: 1.0 ml. of enzyme, 1.0 ml. 1 *M* substrate, total volume 2.3 ml. Temperature 30°. Atmosphere N_2 . Solution acidified with 50% citric acid at 60 minutes.

It is evident that the results are not a conclusive demonstration of the oxalacetate enzyme. The activity on pyruvate was vigorous and may involve the same enzyme as that described by Koepsell, *et al.* (11).

The results from the cell-free juice, in agreement with the results with growing cultures, indicate that the butyl alcohol bacteria are relatively inactive on dicarboxylic acids. Simon and Weizmann (18) have come to the same conclusion. More extensive investigations are needed with cell-free juice under a variety of conditions.

The results which have been presented do not permit a definite conclusion as to the mechanism of fixation of CO_2 by the butyl alcohol bacteria. With the methods used the activity on dicarboxylic acids appears too low to account for the observed vigorous fixation of CO_2 . In view of the results of Utter, *et al.* (20, 21) it is likely that the fixation of CO_2 occurs by $\text{C}_2 + \text{C}_1$ additions.

SUMMARY

The fixation of CO_2 in the carboxyl group of lactic acid has been shown to be a major reaction in the fermentation of glucose by *Cl. butylicum* in the presence of bicarbonate. Attempts to demonstrate the mechanism of this fixation have been inconclusive, in that the activity of these bacteria on dicarboxylic acids was low. Whereas a primary fixation of CO_2 by 3- and 1-carbon addition in a dicarboxylic acid would necessitate that the dicarboxylic acid be converted rapidly to lactate.

It seems possible that there is fixation of CO_2 by 2- and 1-carbon addition but as yet there is no direct evidence for such a reaction.

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On the Mechanism of Enzyme Action. Part 25

Balance of Accumulated Pyruvic Acid (PA) in Carbohydrate Fermentations by *Fusaria*

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INTRODUCTION

Among facts signifying progress in the biochemistry of *Fusaria* noteworthy observations are that (a) contrary to the original considerations of Claude Bernard (1), potassium cyanide may be utilized as a suitable carbon and nitrogen source, (b) pyruvic acid (PA) is the key substance in alcoholic fermentation of hexoses and pentoses without (as in the case of *Chaetomium funicola* (1a)) previous accumulation of phosphoglyceric acid in the presence of NaF, and (c) elementary sulfur may serve as a hydrogen acceptor in dehydrogenations. It was also established that nitrate ions added to hexose- or pentose-containing *Fusaria* cultures were reduced as far as hydroxylamine and it was concluded that the appearance of nitrite ions causes an inhibition of the carboxylase present in these fungi, giving rise to isolable amounts of PA in the course of the degradation of carbohydrates. The amounts of PA isolated when ammonium sulfate was present in the media were considerably smaller (2). These facts were amplified by the observation of Wirth (3) that phosphoglyceric acid was not utilized by *Fusaria* as a carbon source to any measurable extent.

On the other hand, from simultaneous investigations carried out with yeasts or *B. turcosum*, it was deduced (4) that there is a great difference in the permeability of cells for longer, polyvalent anions and shorter substrate anions. Consequently, it would also seem to be conceivable that when carbohydrates and nitrates (or sulfates) are

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supposed to reach the actual location of enzymatic activity in our molds, the rates of penetration will be favorable to the latter. Due to this competition, it is probable that some of the carboxylase present in the *Fusaria* will be inhibited when the step of PA in a hypothetical phase sequence of degradations is reached. This 'blockade' may be partial or temporary and, consequently will give rise to an accumulation of varying amounts of PA.

Experiments will, therefore, be reported which serve as a basis in establishing a balance between the quantity of PA and alcohol formed or obtained under the influence of nitrite, sulfate, hydroxylamine, and cyanide in glucose fermentations by *Fusaria*.

EXPERIMENTAL

Analytical methods. PA was determined quantitatively with 2,4-dinitrophenylhydrazine. (a) Colorimetrically: employing the improved Lu method (5). (b) Gravimetrically: by direct precipitation as described in previous papers from this laboratory. When the colorimetric method was used the requisite dilutions automatically obviated possible interference by the glucose present. The colorimetric method gave good agreement with the gravimetric determinations when the medium under investigation was analyzed by both methods. The range of sensitivity of the former proved to be, however, far higher. For the determination of nitrite the coupling reaction between diazotized sulfanilamide and *N*-(1-naphthyl)-ethylenediamine was used (6). Colorimetric readings were carried out with an Evelyn photoelectric colorimeter using filter 520.

Nitrates were precipitated with nitron as heretofore. Glucose was determined polarimetrically, and alcohol by oxidation with potassium dichromate (7). Mat weights were obtained after filtering the mycelia through porous alundum crucibles and drying at 60°. The *Fusarium* culture employed in this series of experiments was the same as in previous investigations (8).

Procedures. All experiments were carried out in 125 ml. Erlenmeyer flasks containing 50 ml. of medium. In experiments in which certain substances had to be added to the growing organism, three liter Fernbach flasks were used with 750 ml. of medium. Inoculations were made with spores grown on agar plates. Sterilization was effected in live steam on three consecutive days. In experiments carried out in Erlenmeyer flasks, at least the contents of three were filtered on the day of analysis. In the case of Fernbach flasks, portions were removed under sterile conditions. The residual amounts of the media to be analyzed later, were stored in an ice chest at 0°C.

RESULTS AND DISCUSSION

1. Quantitative Relations Between Nitrite Ion and PA Accumulation Using Potassium Nitrate as a Nitrogen Source

In Table I data representing the relation between nitrate disappearance, nitrite accumulated and PA obtained are recorded. It can be

TABLE I
Balance of Nitrate, Nitrite, and Isolated PA

Day	1.00 g. KNO ₃					3.00 g. KNO ₃					5.00 g. KNO ₃				
	KNO ₃ utilized	NO ₂ ' accumulated	PA	Glucose fermented	Mycelial wgt.	KNO ₃ utilized	NO ₂ ' accumulated	PA	Glucose fermented	Mycelial wgt.	KNO ₃ utilized	NO ₂ ' accumulated	PA	Glucose fermented	Mycelial wgt.
	mg.	γ	mg.	g.	mg.	mg.	γ	mg.	g.	mg.	mg.	γ	mg.	g.	mg.
1		2.7					2.7					2.5			
2		1.0	19				9.6	19				29.6	23		
3	27	1.5	29	0.02	44	84	6.3	39	0.06	41	126	12	36	0.09	43
4	48	0.6	53	0.18	89	115	2.5	69	0.25	83	172	3	82	0.22	89
5			77	0.48	165	158	0.6	95	0.41	122	211	1	111	0.49	152
6			88	0.71	169	205	+	120	0.90	234	277	+	143	0.85	205
8			80	1.25	197	287		155	1.49	289	384	+	215	1.56	248
11			74	1.78	214			180	2.07	234			215	3.57	418

Results expressed in g., mg. or γ in 100 ml. of medium.

Nutrient medium: 40.00 g. Glucose, 1.00, or 3.00, or 5.00 g. KNO₃, 5.00 g. KH₂PO₄, 0.75 g. MgSO₄·7H₂O. Water to 1 liter.

TABLE IIa
Influence of Nitrite and Hydroxylamine using Potassium Nitrate as a Nitrogen Source

Day	Blank		10 mg. NaNO ₂		25 mg. NaNO ₂		50 mg. NaNO ₂		100 mg. NaNO ₂		50 mg. NH ₄ OH		100 mg. NH ₄ OH		200 mg. NH ₄ OH	
	PA accu- mulated	Glucose fer- mented	PA accu- mulated	Glucose fer- mented	PA accu- mulated	Glucose fer- mented	PA accu- mulated	Glucose fer- mented	PA accu- mulated	Glucose fer- mented	PA accu- mulated	Glucose fer- mented	PA accu- mulated	Glucose fer- mented	PA accu- mulated	Glucose fer- mented
	mg.	g.	mg.	g.	mg.	g.	mg.	g.	mg.	g.	mg.	g.	mg.	g.	mg.	g.
4	58		55		48		52		54		54		53		58	
5	133		91		93		82		85		104		72		73	
6	144		113		106		108		129		135		108		95	
8	172	1.67	140	1.22	137	1.29	141	1.18	165	1.33	155	1.80	152	1.39	155	1.43
10	169	2.39	145	1.74	163	1.92	148	1.64	190	1.94	140	2.42	180	1.56	195	2.12

Alcohol on 10th day: 666 mg.	501 mg.	527 mg.	470 mg.	497 mg.	643 mg.	548 mg.	566 mg.
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Nutrient medium: 40.00 Glucose, 5.00 g. KNO₃, 5.00 g. KH₂PO₄, 0.75 g. MgSO₄·7H₂O. Water to 1 liter.

TABLE IIb
Influence of Nitrite and Hydroxylamine using Ammonium Sulfate as a Nitrogen Source

Day	Blank		10 mg. NaNO ₂		25 mg. NaNO ₂		50 mg. NaNO ₂		100 mg. NaNO ₂		50 mg. NH ₄ OH		100 mg. NH ₄ OH		300 mg. NH ₄ OH	
	PA ac-mu-lated	Glucose fer-mented	PA ac-mu-lated	Glucose fer-mented	PA ac-mu-lated	Glucose fer-mented	PA ac-mu-lated	Glucose fer-mented	PA ac-mu-lated	Glucose fer-mented	PA ac-mu-lated	Glucose fer-mented	PA ac-mu-lated	Glucose fer-mented	PA ac-mu-lated	Glucose fer-mented
4	7	g.	7	g.	9	g.	7	g.	7	g.	8	g.	8	g.	8	g.
5	16		11		12		8		9		8		2		1	
6	24		19		13		7		7		16		9		2	
8	35	0.36	22	0.34	28	0.12	9	0.12	8	0.35	27	0.35	19	0.36	6	0.30
12	39	0.79	26	0.81	48	0.51	9	0.51	7	0.72	33	0.72	33	0.77	21	0.58

Alcohol on 12th day: 265 mg.	291 mg.	194 mg.	120 mg.	10 mg.	225 mg.	223 mg.	188 mg.
Mycelium on 12th day: 384 mg.	412 mg.	330 mg.	53 mg.	42 mg.	388 mg.	355 mg.	303 mg.

Nutrient medium: 40.00 g. Glucose, 3.2 g. $(\text{NH}_4)_2\text{SO}_4$, 5.00 g. KH_2PO_4 , 0.75 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Water to 1 liter.

readily seen that up to the third day the nitrate disappearance and nitrite accumulation are proportional. From here on, diminution of the nitrite ion exceeds its formation. The amounts of isolated PA and glucose fermented are also related to the nitrate disappearance. In contradistinction to the observation with *P. sclerotiorum* (9) that increasing nitrate concentrations yield reduced mycelium, our analyses with *Fusaria* show the opposite effect.

2. Effect of Added Nitrite and Hydroxylamine on PA Accumulation and Alcohol Formation

These experiments were carried out by adding on the fourth day known amounts of sodium nitrite and hydroxylamine sulfate, respectively, to a basic medium containing potassium nitrate and ammonium sulfate having equal nitrogen values. In distinction to the effect of nitrite ion in the higher concentration and to the effect of hydroxylamine in photoreduction in green algae, the latter did not lessen the growth appreciably nor did it influence the metabolism of the *Fusaria*. However, in good agreement with our earlier (2) postulation concerning ease of formation of isonitrosopropionic acid (10), the amount of PA isolated becomes appreciably smaller soon after the addition of the hydroxylamine, especially in those cases in which the nitrate was replaced by sulfate. In contrast to the effect of nitrite which irreversibly inhibited the course of the reaction, however, the unchecked progress of PA accumulation in presence of hydroxylamine is obvious. The alcohol determinations in all cases in which ammonium sulfate was used instead of potassium nitrate showed an increase in relation to the sugar fermented as contrasted with decreased values signifying isolated PA. This shows the greater inhibitory effect of nitrite ions on carboxylase as compared with the action of sulfate ions.

3. Effect of KCN on the Accumulation of PA

All quantities of PA obtained and isolated in carbohydrate fermentations by *Fusaria* in the presence of nitrites or ammonium sulfate represent a total amount which is derived from the PA obtained through the inhibitory action of the salts mentioned on carboxylase. But, from the standpoint of analytical interpretation, it remains insignificant whether the inhibitions occur through chemical action on the prosthetic group characterized by the NH_2 -group present in its

molecule, or by affecting the carrier protein, or by causing a separation of both. In turn, the nitrate-nitrite reduction may ensue from two reactions. (a) By the action of hydrogen freed by the dehydrogenases and carried over to the acceptor nitrate from hydrogen donors formed in the course of the initial degradation of the hexoses and pentoses present, and (b) by the action of a hypothetical reductase, hitherto regarded as HCN-sensitive, effecting the direct reduction of nitrate to nitrite. Under appropriate experimental conditions it should be, therefore, possible to separate the two modes of action and consequently less PA should be found in media which were prepared with potassium nitrate to which KCN had been added.

TABLE IIIa

Accumulation of PA in Presence of KCN Using Potassium Nitrate as a Nitrogen Source

Day	KCN added			PA accumulated			
	1	2	3	Blank	1	2	3
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
4	20	30	50	119	110	118	96
5	70	80	100	156*	90*	102*	128*
7	70	80	100	168	100	50	95
8				172	79	9	6
9				186	80	10	40

Glucose fermented on 9th day	<i>g.</i>	2.65	2.57	3.10	3.45
Alcohol on 9th day	<i>mg.</i>	763	705	753	534
Mycelium on 9th day	<i>mg.</i>	1067	1113	987	1255

Nutrient medium: 40.00 g. Glucose, 5.00 g. KNO₃, 5.00 g. KH₂PO₄, 0.75 g. MgSO₄·7H₂O. Water to 1 liter.

* Analyzed on the 6th day.

An inspection of the data recorded in Table IIIa shows clearly that, compared with the PA values of the blank, the amounts of PA isolated from experiments which were carried out in the presence of excessive and increasing amounts of KCN, exhibit a steady drop. Despite this observation most of the values representing the amount of glucose

fermented were increasing and the alcohol values closely approach the value obtained in the blank. We know, however, from earlier investigations that this slight diminution of the latter is not due to inhibited fermentation but to subsequent dehydrogenation of the alcohol previously formed. Consequently, it would appear that there is a reductase operative in *Fusaria* which, judging from the amounts of KCN tolerated in these experiments, must be highly HCN-insensitive. A similar enzyme is claimed to be present in *B. pyocyaneus* (11).

TABLE IIIb

Accumulation of PA in the Presence of KCN Using Ammonium Sulfate as a Nitrogen Source

Day	KCN added		PA accumulated		
	1	2	Blank	1	2
	mg.	mg.	mg.	mg.	mg.
4	20	30	21	18	21
5	75	10			
6			24	20	23
7	100	200	33	27	30
8			33	33	33
9			30	40	43

Glucose fermented on 9th day	g.	0.81	0.88	1.08
Alcohol on 9th day	mg.	287	211	199
Mycelium on 9th day	mg.	357	305	457

Nutrient medium: 40.00 g. Glucose, 3.2 g. $(\text{NH}_4)_2\text{SO}_4$, 5.00 g. KH_2PO_4 , 0.75 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Water to 1 liter.

In the experiments carried out with a medium in which potassium nitrate was replaced by ammonium sulfate, the absolute amounts of isolated PA are much smaller and remain unchanged on addition of increasing quantities of KCN. In spite of this the alcohol values are comparable with those of the blank. Since the reductase mentioned above does not act on the ammonium sulfate, only the formation of that amount of PA which results from the slight inhibitory effect of the sulfate on the carboxylase present can be measured. This would

signify that the above mentioned total value of PA consists of only that part which is derived from the direct action of the sulfate ions on the carboxylase in the presence of ammonium sulfate. As neither the reductase nor the hydrogen freed from intermediary hydrogen donors by the dehydrogenases present are capable of reducing ammonium sulfate, there is no possibility that an inhibition of the aforementioned reductase would influence the PA values which, contrary to the 'NO₂'-PA values, remain undiminished when KCN is added to the ammonium sulfate-containing media.

The experiments presented lend further support to the thesis that undistorted results may be obtained in studying the course of microbiological reactions *in vivo* without introducing interceptors. It would also seem that the results are indicative of a prevailing variance with the mechanisms depicting an apparent course ascribed to the action of yeast preparations.

SUMMARY

1. Quantitative experiments were presented which indicate that in addition to nitrite ions, sulfate ions are also capable of measurably inhibiting the action of carboxylase in *Fusaria*.
2. The PA isolated through inhibition of the carboxylase is obtained by the action of hydrogen derived from certain hydrogen donors and by the action of a reductase on nitrate.
3. The reductase of *Fusaria* is highly HCN-insensitive.

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Studies on Histidase *

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INTRODUCTION

Histidase was simultaneously and independently discovered in 2 laboratories in 1926. György and Röthler (1) found that the addition of histidine leads to a large increase in the amount of ammonia formed during sterile autolysis of liver, and Edlbacher (2) obtained a similar increase on incubating histidine with an aqueous liver extract. The enzyme was distinguishable from arginase (3). Besides ammonia, Edlbacher, Kraus, and Scheurich (4) were able to isolate glutamic acid from the reaction product as well as to show the presence of a volatile reducing acid, probably formic acid. This seemingly indicated that the reaction could be expressed by



While the formation of ammonia was confirmed by Abderhalden and Buadze (5), Mislowitzer and Kauffmann (6) obtained only one mole of ammonia per mole of histidine instead of two as would be expected from the above equation. The discrepancy was explained by Edlbacher and Kraus (7) as being due to the type of reagent used to liberate ammonia. When NaOH is used 2 moles of ammonia are obtained while Na_2CO_3 yielded only one (or slightly more) moles. This is evidence that the additional ammonia liberated by NaOH originated from a compound other than the ammonia formed during the hydrolysis of histidine and that actually 2 atoms of nitrogen are in organic combination. The enzyme product was similar to formamide and glutamine with respect to its lability to alkali but considerably more stable to acids. Concentrated HCl was used to isolate glutamic acid, and steam distillation from 20 per cent H_2SO_4 for 3 hours was required to obtain the volatile acid. It is very probable that under these drastic conditions the enzyme product is destroyed.

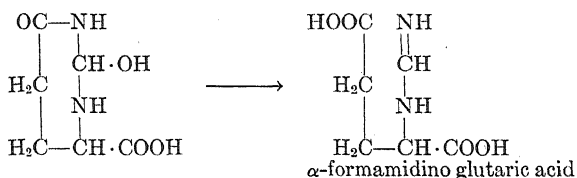
The fact that the glutamic acid isolated was optically active (4) indicates that the ammonia liberated by Na_2CO_3 was derived from a nitrogen contained in the imidazole ring and not from the α -amino group. In the latter case racemic glutamic acid should have been obtained. Further evidence that the ammonia originates from the imidazole group is based on the decreased intensity of the Ehrlich diazo reaction (4) or a modification thereof (8) during the course of the enzyme reaction and that the decreased intensity parallels the production of ammonia. On the other hand it

* Aided by a grant from the Research Board of the University.

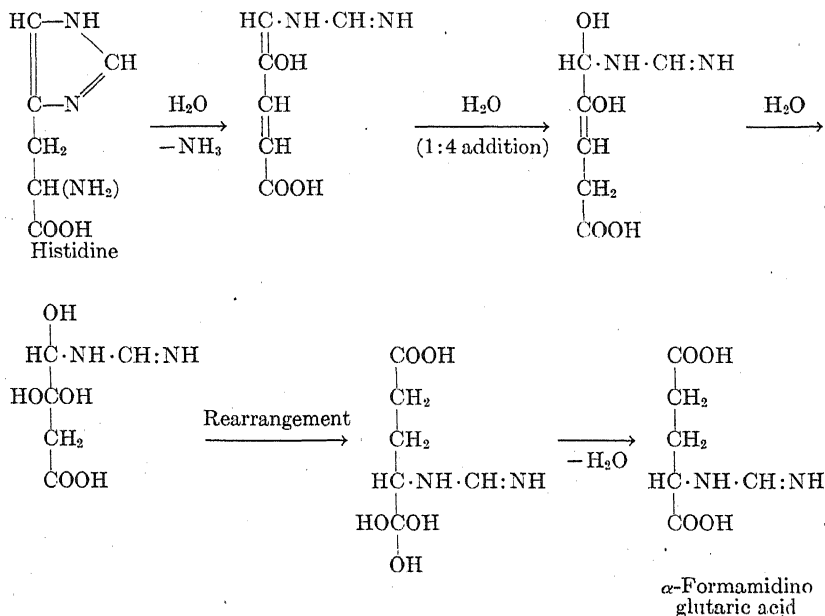
$$\begin{array}{c}
 \text{HC}-\text{NH} \\
 \parallel \quad \diagup \\
 \text{C}=\text{N} \quad \text{CH} \\
 | \\
 \text{H}_2\text{C}-\text{CH}(\text{NH}_2)\text{COOH} \\
 \text{Histidine}
 \end{array}
 \xrightarrow[+\text{2H}_2\text{O}]{-\text{NH}_3}
 \begin{array}{c}
 \text{HC}-\text{NH} \\
 \parallel \quad \diagup \\
 \text{HO}-\text{C} \quad \text{CHO} \\
 | \quad \quad | \\
 \text{H}_2\text{C}-\text{CH}(\text{NH}_2)\cdot\text{COOH}
 \end{array}
 \xrightarrow{\text{Rearrangement}}
 \begin{array}{c}
 \text{HO}-\text{C}-\text{NH} \\
 \parallel \quad | \\
 \text{HC} \quad \text{CHO} \\
 | \quad \quad | \\
 \text{H}_2\text{C}-\text{CH}(\text{NH}_2)\cdot\text{COOH}
 \end{array}
 \longrightarrow
 \begin{array}{c}
 \text{OC}-\text{NH} \\
 | \quad | \\
 \text{H}_2\text{C} \quad \text{CHO} \\
 | \quad | \\
 \text{H}_2\text{C}-\text{CH}(\text{NH}_2)\cdot\text{COOH} \\
 \text{Formyl glutamine}
 \end{array}
 \longrightarrow
 \begin{array}{c}
 \text{OC}-\text{NH} \\
 | \quad | \\
 \text{H}_2\text{C} \quad \text{CH}\cdot\text{OH} \\
 | \quad | \\
 \text{H}_2\text{C} \quad \text{NH} \\
 | \quad | \\
 \text{H}_2\text{C}-\text{CH}\cdot\text{COOH} \\
 \text{Enzyme product}
 \end{array}
 \xrightarrow[+\text{2H}_2\text{O}]{\text{HCl or NaOH}}
 \begin{array}{c}
 \text{COOH} \\
 | \\
 \text{H}_2\text{C} \\
 | \\
 \text{H}_2\text{C}-\text{CH}(\text{NH}_2)\cdot\text{COOH} \\
 \text{Glutamic acid}
 \end{array}$$

¹ Named from the analogy to formamide ($\text{O}=\text{CH}-\text{NH}_2$), formamido ($\text{O}=\text{CH}-\text{NH}-$) and formamidine ($\text{HN}=\text{CH}-\text{NH}_2$).

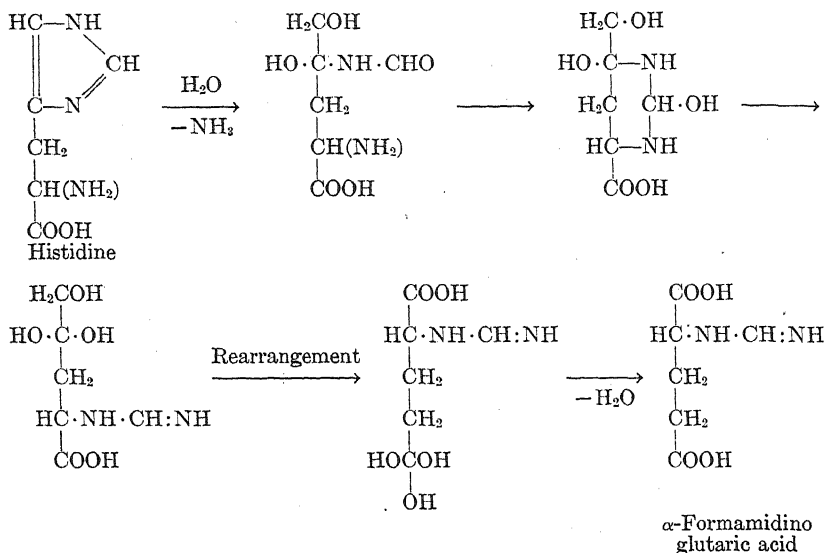
enzyme product postulated by Edlbacher and Neber (10) by rupture of the ring and rearrangement:



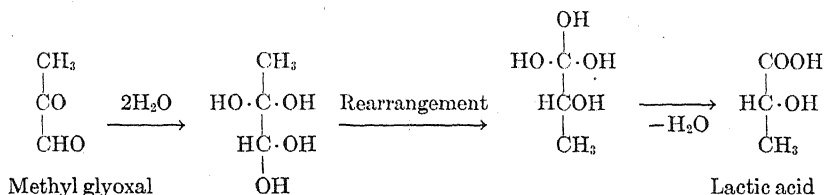
It is necessary to identify the nitrogen atoms in the enzyme products with those in histidine. Edlbacher and Neber's (10) postulate that the glutamic acid nitrogen originates from the α -amino group of histidine is based on the fact that the glutamic acid isolated was optically active. This reasoning may be questioned since Edlbacher and Viollier (11) found that *l*(+)-glutamic acid is formed by the action of urocanase on urocanic acid and subsequent treatment with strong acid. If the glutamic acid nitrogen originates from one of the imidazole nitrogens of histidine, by making use of the benzoin rearrangement, the following enzyme reaction may be postulated:



On the other hand if the α -amino group of histidine becomes the α -amino group of glutamic acid, the following modification of the reaction postulated by Edlbacher and Neber (10) might take place, again making use of the benzoin rearrangement:



A similar benzilic acid type of rearrangement may be applied to the glyoxalase reaction which is the only known enzyme reaction besides that of histidase involving internal oxidation-reduction (other than simple hydration of an ethylene bond):



Hammett (12) considers the base catalyzed reaction of glyoxal to be a benzilic acid rearrangement.

It is apparent from the above that our knowledge of histidase and its mode of action is far from complete. While the present study does not answer all of the questions, it nevertheless throws some additional light on the problems.

EXPERIMENTAL

Histidase apparently occurs only in vertebrate liver (2). Cat liver is the best source (4). This statement is probably based on the experiments of Engeland (13) which showed that more subcutaneously injected histidine is excreted by the rat than by the cat. The following approximate and comparative values of histidase activities, in terms of cat liver histidase, were calculated from the data given by Edlbacher and von Bidder (8): cat, 100; guinea pig, 42; rabbit, 29; pigeon, 42; rat, 35-40. The activity of none of the histidase preparations from the above sources except that from rat liver is inhibited by pyruvate ions (14). Edlbacher, Kraus, and Scheurich (4) extracted the enzyme by grinding the fresh tissue with sand, shaking the pulp with water, and then filtering or centrifuging the mixture. The extract is buffered to pH 8 and used at once. Equally good results were obtained by extracting the pulp with 0.067 *M* phosphate buffer at pH 8. The use of a glycerol extract has been advocated (15) but there appears to be no distinct advantage. Edlbacher, Kraus, and Scheurich (4) were unsuccessful in their attempts to precipitate cat liver histidase in such a way that it could be redissolved to give an active solution. Some impurities could be removed by adsorption on kaolin without loss of enzyme activity. One adsorption of the enzyme on alumina at pH 7.1 and elution with phosphate buffer at pH 8 yielded an active preparation but all loss of activity occurred on repetition of the process. The best preparation showed an increase in purity (the ratio of activity to nitrogen content) of 150% and a decrease in activity per ml. of 75% from the original water-liver mixture. The final preparation contained about 0.2% protein. While this preparation is suitable for qualitative purposes, particularly for the purpose of identifying different enzymes that may be present in the mixture, it is not adapted for quantitative studies on account of its dilution. This necessitates the use of large volumes. Moreover, dilute protein solutions are unstable. The available data indicate that it is easier to free certain of the enzymes present in liver from histidase than the converse (3, 11).

The method of precipitating enzymes with concentrated salt solutions has come into general use. Ammonium sulfate is not suitable in the present instance since ammonia is one of the end-products. Preliminary experiments with $MgSO_4$ and acetone confirmed the unsatisfactory results of Edlbacher, Kraus, and Scheurich (4). Sodium citrate, lithium chloride, potassium oxalate, potassium acetate, and dioxane either failed to precipitate the enzyme or inactivated it. Active precipitates were obtained by the use of strong solutions of potassium phosphate (16) and of citrate (17) adjusted to near neutrality. Greenstein (18) has shown that the proteins contained in crude rat liver extract are nearly completely denatured in the presence of strong solutions of most salts. This observation has been confirmed. Of many salts tried, the only ones without such action were potassium phosphate and various ammonium salts (sulfate, chloride, bromide, acetate, iodide, thiocyanate, and carbonate).

In the present experiments potassium phosphate was employed as "salting-out" agent. Strong buffering at a suitable and constant pH may be attained. The yield of histidase is high. It has the disadvantage that solutions of the strength required to salt out histidase have a density of 1.2-1.3 making the centrifuging of a fine precipitate difficult or impossible. Cat liver frozen at -17° under toluene is thawed

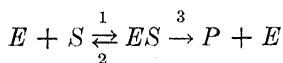
at room temperature and immediately passed through a meat grinder. Satisfactory results were obtained when the liver was stored for 4 months at the low temperature. The ground tissue is extracted with 5 parts by weight of distilled water, preferably but not necessarily at 0-5°. After shaking the mixture for several hours it is filtered on a Büchner funnel with the aid of Supercel. This requires 6 to 8 hours. A clear red filtrate is obtained. This extract has a similar activity and nitrogen content as that of Edlbacher, Kraus, and Scheurich (4). It has a pH of 5.5 to 6.0. To each 100 ml. of the extract 52 g. of phosphate buffer, prepared by dissolving 3.42 moles K_2HPO_4 and 0.38 moles of KH_2PO_4 in water and diluting to one kg., are added and the mixture is placed in the icebox over night. The precipitate is filtered off with the aid of Supercel. The caked precipitate is washed several times with a mixture of 100 ml. water and 60 g. phosphate buffer. Under the conditions used, the enzyme is contained in the least soluble 40% of the water-soluble liver proteins. The crude extract represents about 35% of the total liver protein. Use of the phosphate buffer adjusts the pH to 8.0, at which point the enzyme has maximum stability.

The reprecipitated enzyme extract gives a positive biuret, Millon, Hopkins-Cole, and a negative Molisch test. The Dische test for nucleic acid was faintly positive. Use of the Seibert (19) modification of the Dische test showed that 0.5% of the organic matter was nucleic acid. The nucleic acid could be almost entirely removed by the addition of a large excess of protamin. A small amount of lipid is associated with the protein. The fat-free protein contained 17.7% nitrogen determined according to Koch and McMeekin (20) and 0.08% phosphorus. The latter can be largely accounted for as nucleic acid. The protein precipitates from aqueous solution in the presence of strong phosphate buffer in the form of small white particles too small for ordinary microscopic observation. At a concentration of about 0.4% protein, the solution containing the reprecipitated enzyme has a brownish yellow color which becomes almost black when the protein concentration is increased to 4%. The stronger solutions appear cloudy in reflected light but clear in transmitted light. Traces of hemoglobin were identified by spectroscopic examination.² The majority of the absorption was diffuse, spreading through the blue region. The protein contained 0.15% of iron determined according to Saywell and Cunningham (21). This quantity is larger than can be accounted for by the amount of hemoglobin or catalase present and, since cat liver does not contain ferritin (22), it is probably a constituent of the heat labile protein of the type mentioned by Kuhn, Sörensen, and Birkhofer (23). The reprecipitated material can be dialyzed at 5° for 7 days

² Kindly carried out by Dr. G. Mackinney.

against either 0.036 *M* potassium phosphate buffer at pH 7.6 or 0.05 *M* KHCO₃ without denaturation or considerable loss of enzyme activity. Evidently histidase contains no loosely combined coenzyme as has been suggested by Edlbacher, Baur, and Köbner (24). Their observation that dialysis against water for several days causes complete and apparently irreversible protein denaturation has been confirmed. Addition of NaCl will not dissolve the protein. The pH decreased to 5.8 during the dialysis. This is approximately the isoionic point of the proteins present (25). The pH value is uncertain due to the small amount (0.1%) of protein left in solution. It is probable that the acidity together with the low salt concentration led to denaturation of the proteins. Edlbacher, Kraus, and Scheurich (4) state that preparations partially denatured in this manner show greater enzymic activity than the sum of the activities of the precipitate and the supernatant when measured separately. They consider this as evidence that histidase contains two or more components. However, apparently no controls were carried out to test the possibility that, due to a higher protein concentration, the mixture is more stable than the fractions alone. No evidence for the plural nature of histidase was obtained in the present experiments.

Before proceeding to a consideration of the histidase activities of the various preparations tested and the kinetics of the reaction it is necessary to present the underlying theory. When no inhibitory reactions are taking place, the mechanism of action of many enzymes can be represented by the equation



where *S* represents the substrate, *E* the enzyme, *ES* the enzyme substrate compound, and *P* the products of the reaction. This mechanism implies that the total amount of enzyme (ΣE) and substrate added (*A*) are constant, the rate of formation of *P* and *S* is proportional to *ES*, and the rate of destruction of *S* is proportional to *E* and *S*. These conditions are given by:

$$\Sigma E = E + ES \quad (1)$$

$$A = ES + S + P \quad (2)$$

$$dP/dt = k_3(ES) \quad (3)$$

$$-dS/dt = k_1(S)(E) - k_2(ES) \quad (4)$$

E is eliminated from equation (4) by use of equation (1) and S by the use of equation (2) giving

$$dES/dt + dP/dt = k_1(\Sigma E - ES)(A - P - ES) - k_2ES \quad (5)$$

ES may be eliminated from equation (5) by the use of equation (3):

$$\frac{1}{k_3} \frac{d^2P}{dt^2} + \frac{dP}{dt} = k_1 \left(\Sigma E - \frac{dP}{k_3 dt} \right) \left(A - P - \frac{dP}{k_3 dt} \right) - \frac{k_2 dP}{k_3 dt} \quad (6)$$

On collecting terms and rearranging:

$$\frac{d^2P}{k_3 dt^2} + \frac{k_1 dP}{k_3 dt} \left[\Sigma E - \frac{dP}{k_3 dt} + \frac{k_2 + k_3}{k_1} + A - P \right] - k_1(\Sigma E)(A - P) = 0 \quad (7)$$

The term $\frac{d^2P}{k_3 dt^2}$ represents the rate of change of ES . It should be a negligible quantity except possibly at the very beginning of the reaction since ES is present in small amounts. The term $\Sigma E - \frac{1}{k_3} \frac{dP}{dt}$ is equal to E and, in all known conditions, is negligibly small in comparison to $\frac{k_2 + k_3}{k_1}$. Elimination of these terms gives:

$$\frac{dP}{dt} \left[\frac{k_2 + k_3}{k_1} + A - P \right] - k_3(\Sigma E)(A - P) = 0 \quad (8)$$

Integration with the limiting condition of $P = 0$ when $t = 0$ gives:

$$k_3(\Sigma E)t = P + \frac{k_2 + k_3}{k_1} \ln \left[\frac{A}{A - P} \right] \quad (9)$$

This derivation is similar to that of Briggs and Haldane (26) except that no assumptions need be made about the size of $A - P$. Except under special circumstances, there is no experimental way of determining values for k_1 and k_2 . Following Michaelis and Menten (27) the term $\frac{k_2 + k_3}{k_1}$ will be designated by K_m but without any assumptions about the relative size or physical significance of the constants involved. K_m , A , and P are expressed in terms of moles of substrate per liter. The term $k_3 \Sigma E$ will be designated as V_m . This term is a measure of the concentration of enzyme present in moles of substrate per liter

per hour when t is expressed in hours. Substituting these terms in equation (9) gives:

$$V_m = P + K_m \ln \left[\frac{A}{A - P} \right] \quad (10)$$

This equation will represent the entire course of an enzyme reaction as represented above. It will be shown later that the histidase reaction follows equation (10) within the limits of error. At 37° and pH 7.8, K_m is shown to be 0.001 moles of histidine per liter. Measurement of A , P , and t will therefore determine the concentration of enzyme as measured by V_m . The quantity $V_m q$, where q is the volume of solution in ml., will be a measure of the amount of enzyme present.

When the experimental conditions are so adjusted that A is much larger than $V_m t$ and K_m , the term $K_m \ln \left[\frac{A}{A - P} \right]$ may be neglected.

This gives

$$V_m t = P \quad (11)$$

Measurement of P and t is all that is needed for the determination of V_m . This is the basis for the determination of histidase used by Edlbacher, Kraus, and Scheurich (4). In this determination $A = 0.02$ moles per liter and $t = 6$ hours. The amount of enzyme was so chosen that the value of P obtained was less than 0.008 moles per liter. At this value $K_m \ln \left[\frac{A}{A - P} \right]$ is equal to 0.0005, and hence the error in the value obtained for V_m is less than 6%.

In the procedure of Edlbacher, Kraus, and Scheurich (4) the concentration of P is measured by the amount of ammonia produced on aeration with strong alkali, 2 molecules of NH_3 being equivalent to one molecule of histidine split. In order to avoid the liberation of ammonia from the acid amide groups that may be present in the protein, the solution was deproteinized with metaphosphoric acid. This procedure eliminated the necessity of carrying out a blank determination with its inherent uncertainties when purified histidase was used. Five ml. of a 0.08 M $\text{l}(-)$ -histidine solution of pH 8 are mixed with the histidase solution and the mixture is diluted to 20 ml. The enzyme solution generally has phosphate buffer of pH 8 present. If not, 2 millimoles of buffer are added. Toluol or chloroform are added to saturation. The presence of these compounds, cresol red, or variations in the amount of phosphate buffer (less than required for salting out) does not affect the activity of the enzyme. The mixture is incubated at 38° for 2 hours. Longer incubation periods are used for weakly active enzyme preparations. One ml. of glacial acetic acid and 1 ml. of NaPO_3 solution (1:4) are added to the mixture, the volume brought to 25 ml. and, after shaking, the contents of the flask are filtered. Twenty ml. of the filtrate are aerated for 90 minutes at room temperature, after addition of 5 ml. of 45% NaOH solution,

into standard acid. The excess acid is titrated with $M/50$ NaOH using a mixed indicator of methyl red and methylene blue. Blank determinations on the histidine solution and on the deproteinized enzyme solution yielded insignificant amounts of ammonia.

When a 2 hour incubation period is used the number of histidase units, HU , present is $0.417 \times$ the difference in titer from the blank in ml. of $M/50$ NaOH. The factor includes a volume correction of $25/20$ to compensate for the dilution. This calculation gives a histidase unit equivalent to the " $(HE)_2$ " of Edlbacher, Kraus, and Scheurich (4). Their finding that the enzyme activity decreased in a 6 hour incubation was not confirmed. If incubation periods longer than 2 hours are used the results may be reduced to this unit by dividing by one half the time employed. The limit of 16 ml. $M/50$ NaOH should be observed. If larger amounts of NH_3 are produced or for accurate work when the amounts are less, the data should be corrected by the use of equation (10). With the determination as given above, one HU is equal to a Vmq of 0.015 mM histidine per hour. The histidase activity of the crude liver extract was $1.1 HU$ per millimole nitrogen. Further values were obtained as follows: 1st precipitation, 2.4; 2nd precipitation, 2.6; 3rd precipitation, 2.7. The absolute activity of the enzyme may be estimated roughly from the latter value if it is assumed that the enzyme is pure and the molecular weight is 90,000. Then the turnover rate (28) of histidase is 760 moles histidine per mole protein per minute.

In the present method of the partial purification of histidase, liver esterase, arginase, and the various dehydrogenases were almost completely removed. Catalase is present in the same material as histidase. The partially purified histidase preparation gave a *Kat. f.* value of 300 to 600 when determined according to Sumner (29).

Electrophoresis Studies. The livers of three cats were perfused with warm Locke's solution under ether anaesthesia by cannulation of the portal vein. Perfusion was carried out for one hour at an average rate of 50 ml. per minute. Hemoglobin was almost completely removed and probably considerable amounts of soluble liver protein as well. A crude liver extract was prepared and a portion subjected to salt precipitation in the manner given previously. The crude and the purified preparations were dialyzed to equilibrium against phosphate buffer of ionic strength 0.10 and pH 7.75 for 8 days at 6° . The osmotic pressure of the purified preparation corresponded to a molecular weight of 60,000. The true molecular weight is probably several times the above value since the solution was 2 pH units removed from the isoelectric point. The difference in pH inside and outside the dialysis bag was less

than 0.05 units; the pH could not be determined with sufficient accuracy for more accurate calculation of the molecular weight.

The electrophoresis³ experiments on the crude and purified preparations were carried out in the Tiselius cell using the Philpot-Svensson

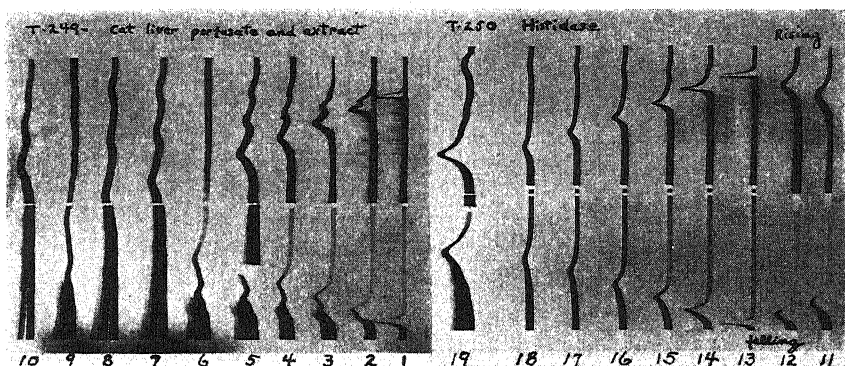


FIG. 1

Electrophoresis Patterns of Crude Liver Extract and Purified Histidase

Crude liver extract:

1. Before start of electrophoresis.
2. 1000 sec. after start.
3. 2000 sec.
4. 3000 sec.
5. 4100 sec. (mostly rising boundary).
6. 5000 sec. (falling boundary).
7. 6000 sec. (rising boundary).
8. 7000 sec. (rising boundary).
9. 8000 sec. (falling boundary). Reversed current at this time.
10. 11,000 sec.
11. 15,000 sec.
12. 16,000 sec.

Purified histidase:

13. Before start of electrophoresis.
14. 1000 sec. after start.
15. 2000 sec.
16. 3000 sec.
17. 4000 sec.
18. 5000 sec.
19. 5500 sec. At a slit angle of 40° instead of 10° as in the other cases.

³ The electrophoresis patterns were kindly prepared for us by Dr. H. P. Lundgren and the staff of the Protein Division of the Western Regional Laboratory of the U. S. Dept. of Agriculture.

optical system (30). The results are shown in Fig. 1. The enzyme is present in the main component of the crude and purified preparation. Its mobility is -5.7×10^{-5} sq. cm. sec^{-1} volt^{-1} at 1° and pH 7.75. Some impurities are present in the "purified preparation." Another run was carried out on a reprecipitated preparation of the enzyme. The falling boundary is shown in Fig. 2. The preparation consists of a single electrophoretic component. The asymmetry of the boundary indicates a small amount of impurity. No separation of the proteins present could be achieved at the pH employed. The wide shape of the

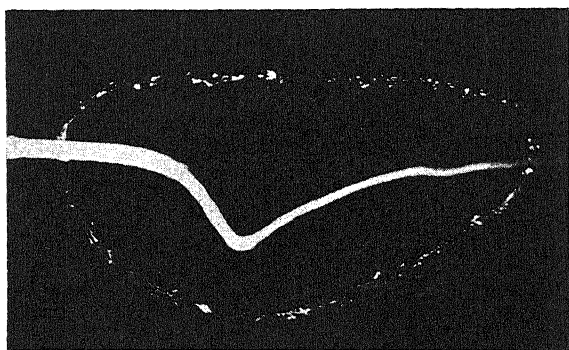


FIG. 2

Enlargement of Schlieren Boundary During Electrophoresis of Purified Histidase
Positive exposure instead of negative as in Fig. 1.

peak is of the type that, in general, characterizes solid solutions of proteins.

Phase Rule Studies. When a single amount of protein is dissolved to varying ionic strengths, a type of solubility curve such as those reported by Jameson (17) is obtained. When a new solid phase appears there will be a change in direction of the curve. According to Jameson "these solid phases must be individual proteins, solid solutions of one or more proteins in another, or continuous series of compounds." This type of study has theoretical drawbacks but it possesses the practical advantage of showing directly the amount of salting-out agent that should be used for a given preparation. Caution must be used in interpretation since results obtained with varying amounts of protein may be different.

The results of such an experiment are represented graphically in

Fig. 3. Ten ml. of the purified histidase preparation were introduced into a flask and phosphate buffer in such amounts as to give the ionic strength indicated. Precipitation began immediately. After standing in the icebox overnight, the mixtures were passed through

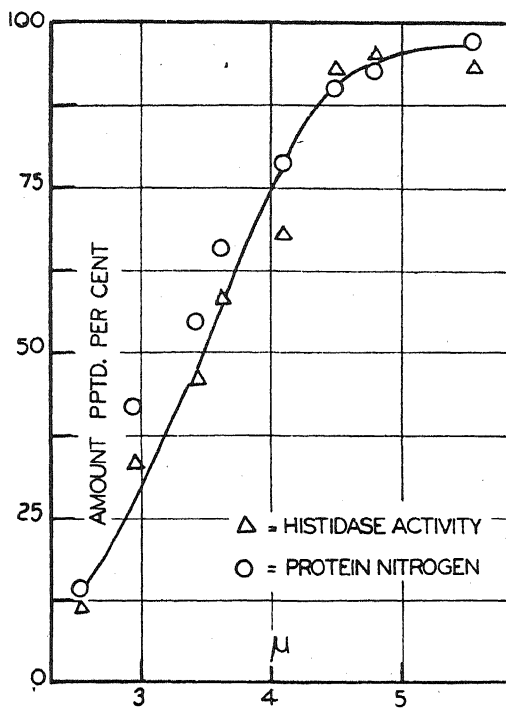


FIG. 3

Solubility of Histidase in Concentrated Phosphate Buffers: Effect of Varying Phosphate Concentration with a Constant Amount of Histidase

Solution of reprecipitated histidase precipitated with amounts of concentrated solution of 9 K_2HPO_4 :1 KH_2PO_4 sufficient to give the ionic strength, μ , in moles per kilogram H_2O given on abscissa. $t = 25^\circ$.

Whatman No. 5 filter paper with suction, returning the filtrates until they were perfectly clear. The precipitates were sucked nearly dry and then washed through the filters with distilled water. Activity measurements were carried out on these filtrates. The metaphosphate precipitate of protein removed before carrying out the ammonia deter-

mination was washed on the filter with 5% trichloroacetic acid. Micro-Kjeldahl determinations were carried out on the washed filters.

Within the limits of error, no discontinuity in precipitated protein nitrogen or histidase activity was observed over the entire range of amounts of salt added. The protein nitrogen and histidase activity run parallel, showing that no further fractionation of the material with respect to histidase activity can be achieved by use of this precipitant. The salting-out constant K_s , has a value of 0.52. In terms of liters (instead of kilograms of water), K_s' has an estimated value of 0.60, assuming that the change in density of the phosphate solutions is proportional to the concentration. This value is considerably smaller than for other proteins (31).

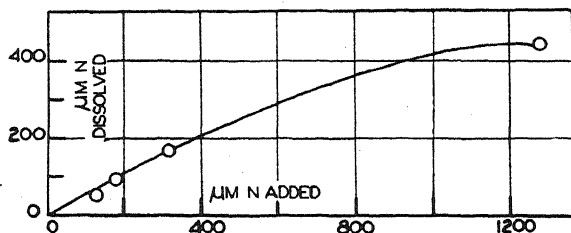


FIG. 4

Solubility of Histidase in Concentrated Phosphate Buffers: Effect of the Amount of Protein Nitrogen Added on the Amount of Protein Nitrogen Dissolved
Solvent: 9 K_2HPO_4 :1 KH_2PO_4 ; pH = 8.0; $\Gamma/2 = 2.5$; $t = 5^\circ$. Varying amounts of solution of reprecipitated histidase that contained the amounts of protein nitrogen given were added. Total volume of each solution, 25 ml.

Similar results were obtained with the crude enzyme extract and on a preparation salted out only once. In these cases the slopes are the same, but the whole curves are displaced to higher ionic strengths. This is a common phenomenon; the solubility of a protein decreases with removal of soluble impurities. In the crude liver extract the protein curve continues upward at a new slope after all of the histidase has been precipitated.

The second type of phase rule study carried out consisted in studying the effect on solubility of varying the amount of protein added to a solvent of constant composition. This method has been frequently used by Northrop and coworkers (32). It is quite suitable for determining the homogeneity of a protein and, in general, it is more sensi-

tive than either electrophoresis or ultra-centrifugation. The results of such an experiment on reprecipitated histidase are represented graphically in Fig. 4. Quantities of this preparation containing the amounts of protein given on the abscissa were added to an amount of phosphate buffer and diluted to a volume such that the final ionic strength ($\Gamma/2$) was 2.5 moles per liter. After standing in the icebox for 2 days, the mixtures were filtered and the nitrogen contents of the filtrates determined. The results are typical for a solid solution. The amount of enzyme in solution increases with the amount of solid phase present, showing that the latter does not have a fixed composition.

When, in such an experiment, the enzyme activity is measured, information only about proteins possessing the measured activity is obtained. The following experiments were carried out with a purified

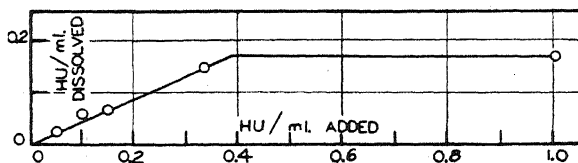


FIG. 5

Solubility of Histidase in Concentrated Phosphate Buffers: Effect of Amount of Histidase Activity Added on the Amount of Histidase Activity Dissolved
Varying concentrations of reprecipitated histidase in a solvent containing 0.03 M $KHCO_3$ and 0.54 M mixture of 9 K_2HPO_4 and 1 KH_2PO_4 . $\Gamma/2 = 1.49$; $t = 25^\circ$.

histidase preparation that had been thoroughly dialyzed against 0.05 M $KHCO_3$ solution. Various amounts of this preparation were added to a constant amount of phosphate buffer of pH 8.0 and diluted to a constant volume with 0.05 M $KHCO_3$. The final concentration of phosphate was 0.54 mole per liter. The results are shown graphically in Fig. 5. The enzyme units dissolved increased less rapidly than the number of units added. This might be considered as evidence that there is more than one component with histidase activity or, what appears more likely, that inactivation took place in the course of the experiment.

Kinetics of Histidase Reaction

(a) *Inhibition.* The competitive inhibition of histidase was studied by Edlbacher, Baur, and Becker (14). Of the many nitrogenous bases that are competitive inhibitors, the strongest are imidazole, histamine,

and *d*(+)-histidine. The affinity of the latter compounds for histidase is about equal to that of *l*(-)-histidine. Imidazole acetic acid and ethylene diamine have about half this affinity. Thirteen other bases inhibited the enzyme still less. Sodium pyruvate is also an inhibitor (14, 24). Since this could only be shown with very fresh rat liver, it seems likely that pyruvate serves as a substrate for other enzyme reactions that lead to the fixation of ammonia formed from histidines. Like many other enzymes, histidase is inhibited by heavy metallic ions. Copper, cadmium, and zinc inhibit the activity of crude liver extract at a concentration of 10^{-4} *M*. Application of the method of Easson and Stedman (33) for the determination of the absolute activity of an enzyme to histidase is not feasible since it would require some competitive inhibitor with an affinity for the enzyme at least 1000 times greater than any known.

In the present studies the pH of the compounds (the final molar concentrations are given in parentheses) tested for inhibitory action was adjusted to 8. Determination of histidase activity with and without inhibitor were carried out and the percentage inhibition calculated. The following results were obtained: Na_2SO_3 (0.05), 10; dimedone (dimethylcyclohexanedione) (0.01), 12; thiourea (0.01), 13; Na_2S (0.05), - 39; KCN (0.05), 84; $\text{Na}_4\text{P}_2\text{O}_7$ (0.12), 0; semicarbazide (0.001), 0; hydroquinone (0.01), 47; CO (sat.), 0. Potassium cyanide has a marked inhibitory action. Presumably the concentration used is considerably greater than employed by Edlbacher and von Segesser (34) who state that cyanide has no effect on histidase. The effect found is apparently not due to combination with a heavy metal necessary for enzyme activity since thiourea, CO, and pyrophosphate showed no such effect. The alternative possibility that cyanide combines with some carbonyl group necessary for enzyme activity as in the case of diamine oxidase (35) is also eliminated by the lack of significant effect of semicarbazide and dimedone. Sodium sulfide had an accelerating effect at 0.05 *M* concentration. Edlbacher, Baur, and Köbner (24) could find no effect with cysteine. In the case of hydroquinone it appears likely that the inhibitory action was due to the quinone which could be seen forming by oxidation with air during the course of the enzyme reaction.

(b) *Effect of pH on the Histidase Reaction.* Edlbacher (2) reported a sharp optimum for histidase activity at pH 9.1. Abderhalden and Buadze (5) found the enzyme to be 50% more active at pH 9.3 than

at 8.3. Later Edlbacher, Kraus, and Scheurich (4) reported a broad optimum zone between pH 8 and 9.

In the present experiments the activity of histidase was determined at various pH values in the presence of excess substrate. Enzyme activities were carried out in the manner given previously except that buffer and acid or base were added to the reaction mixture before addition of the enzyme. There was no detectable change in pH during incubation of the samples. Tests were carried out with and without various added buffering agents. No discontinuities in the curves were found which makes it probable that changes in pH rather than changes

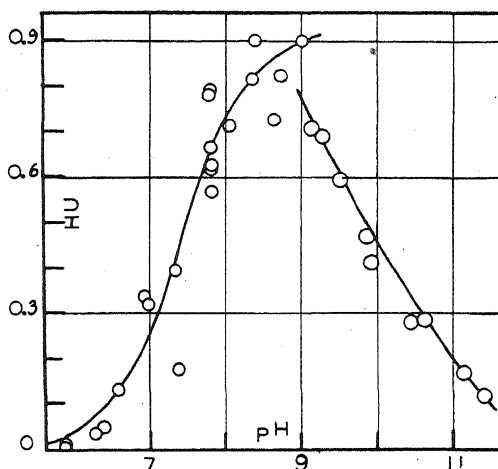


FIG. 6

Effect of Acidity on the Measured Activity of a Solution of Reprecipitated Histidase
Initial histidine concentration = 20 mM per liter; $t = 38^\circ$.

in buffering salts are responsible for the effects found. The data are represented graphically in Fig. 6.

There is a sharp optimum in histidase activity at pH 8.5. The acid side of the curve is considerably steeper than reported by Edlbacher, Kraus, and Scheurich (4) and the alkaline side is slightly steeper. The purified preparation appears to be more sensitive to pH than the crude liver extract.

Reversible effects of pH on enzyme activity may be attributed to ionization of some group in either the enzyme, E , the substrate, S , or the substrate complex, ES , if the ionization makes the component or

components of the reaction no longer capable of reacting. The components are assumed to ionize according to the equation

$$K_x = \frac{(H^+)(X)}{(HX)} \quad (12)$$

where X represents either S , E , or ES , and HX is the inactive form involved. Equation (1) and (2) may now be written:

$$\Sigma E = E + HE + ES + HES \quad (13)$$

$$A = ES + HES + S + HS + P \quad (14)$$

Substitution of equation (12) into equation (13) and (14) gives:

$$\Sigma E = E \left(1 + \frac{(H^+)}{K_E} \right) + ES \left(1 + \frac{(H^+)}{K_{ES}} \right) \quad (15)$$

$$A = ES \left(1 + \frac{(H^+)}{K_{ES}} \right) + S \left(1 + \frac{(H^+)}{K_S} \right) + P \quad (16)$$

These equations are analogous to equations (1) and (2). If they are solved in the manner previously used, equation (17) is obtained instead of equation (10):

$$\frac{1}{\left(1 + \frac{(H^+)}{K_{ES}} \right)} V_{mt} = P + \frac{\left(1 + \frac{(H^+)}{K_E} \right) \left(1 + \frac{(H^+)}{K_S} \right)}{\left(1 + \frac{(H^+)}{K_{ES}} \right)} K_m \ln \left[\frac{A}{A - P} \right] \quad (17)$$

If X is the inactive form, each $\left(1 + \frac{(H^+)}{K_X} \right)$ term would be replaced by $\left(1 + \frac{K_X}{(H^+)} \right)$. At any given pH value,

$$\frac{V_m}{\left(1 + \frac{(H^+)}{K_{ES}} \right)} = V'_m, \quad \text{and} \quad \frac{\left(1 + \frac{(H^+)}{K_E} \right) \left(1 + \frac{(H^+)}{K_S} \right)}{\left(1 + \frac{(H^+)}{K_{ES}} \right)} K_m = K'_m$$

When $A \gg V'_m$, K'_m , then:

$$\frac{V_{mt}}{\left(1 + \frac{(H^+)}{K_{ES}} \right)} = P \quad (18)$$

It was found that the acid branch of the curve fits this equation very closely, indicating that as result of the increase in acidity an inactive form of the enzyme-substrate complex is formed. K_{ES} has a value of 7.4. This value probably corresponds to the ionization of an imidazole or a phosphate group. In the event that the imidazole ring is involved, combination with the enzyme has changed the pK' value from 6.1 to 7.4. The alkaline branch of the curve cannot be represented by equation (14). It seems unlikely that this is due to a large increase of K'_m at higher pH values. More likely possibilities are that alkali

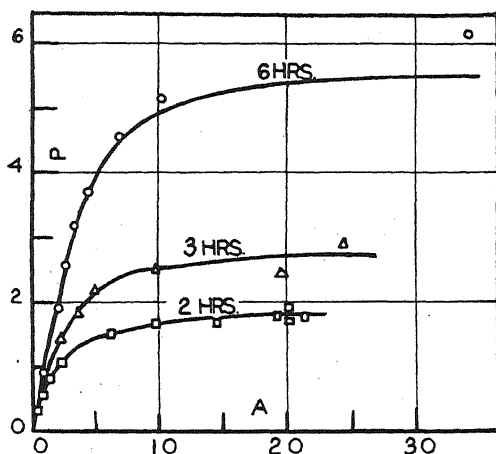


FIG. 7

Effect of Histidine Concentration on Histidase Activity

A = Initial histidine concentration in mM per liter. P = Decrease in histidine concentration in mM per liter after incubation for various periods of time with a histidase solution containing 1.20 HU. pH = 7.8; $t = 38^\circ$.

leads to an irreversible change or that there are two or more components, such as different ionic forms, of the enzyme-substrate complex that are active. These would be expected to dissociate to inactive forms at different pH values.

(c) *Effect of Substrate Concentration and Time of Incubation.* The amount of hydrolysis taking place was measured by using a constant amount of enzyme with varying amounts of substrate and varying reaction times. The data are shown graphically in Fig. 7. Enzyme containing 0.495 millimoles of protein nitrogen and 1.20 histidase units

were present in the reaction mixture. The total volume was 20 ml., the pH 7.8, and the incubation temperature 38°. Histidine in the amounts indicated was added. Ammonia determinations were carried out as stated previously.

Equation (10) may be arranged to the following form:

$$\frac{1}{t} \left[\ln \frac{A}{A-P} \right] = \left(-\frac{1}{K_m} \right) \frac{P}{t} + \frac{V_m}{K_m} \quad (19)$$

A plot of $\frac{1}{t} \ln \left[\frac{A}{A-P} \right]$ against $\frac{P}{t}$ will give a straight line that has a slope of $-\frac{1}{K_m}$ and an intercept on the $\frac{P}{t}$ axis of V_m (Fig. 8). The best

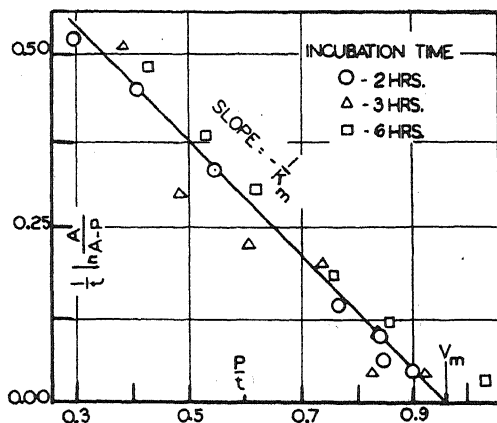


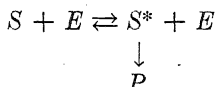
FIG. 8

Calculation of Constants of the Michaelis-Menten Equation from the Data Given in Fig. 7

values of these constants were calculated, treating the equation as a regression of $\frac{P}{t}$ on $\frac{1}{t} \ln \left[\frac{A}{A-P} \right]$ (36). Values were omitted when an error in P of 0.005 millimole per liter (near the limit of error of the method) would have caused a 20% error in $\ln \left[\frac{A}{A-P} \right]$. The numerical constants found were: $K_m = 1.21 \times 10^{-3}$ molar histidine and $V_m = 0.959$ moles histidine per liter per hour.

The curves in Fig. 7 were calculated from these constants. No systematic deviation from the theoretical equation was observed. The

data cannot be represented by either a zero or a first order equation alone. Thus the kinetics are consistent with the mechanism given previously. The same type of reaction might also result from a chain mechanism of the type suggested by Moelwyn-Hughes (37). However, this author suggests that this mechanism probably only applies to reactions with a K_m value of about 10^{-6} . Reactions of the type



where S^* is an activated form of the substrate, are ruled out since the kinetics would be only of the first order. No evidence is available as to whether or not the reaction is reversible. If it is, the equilibrium mixture must contain less than 2% of histidine.

It had been expected that the enzyme would show considerably less activity per hour at 6 than at 2 hours because of slow denaturation of the enzyme during incubation. From crude data of Edlbacher, Kraus, and Scheurich (4) the loss had been estimated to be 10–30%. However, no such effect was observed. There was no significant difference between the values for V_m and K_m at different incubation periods. The purification probably removed substances destructive to histidase.

(d) *Effect of Enzyme Concentration.* A test of the effect of enzyme concentration on the activity of the enzyme was carried out. Varying amounts of enzyme were incubated for 2 hours with 0.4 millimoles of histidine. The amounts of enzyme added, in terms of millimoles of protein nitrogen, are given in parentheses together with the corresponding V_m values (moles histidine/moles protein nitrogen per hour): (1.21) 0.312; (0.61) 0.320; (0.24) 0.297; (0.12) 0.265. The data indicate that, except for the lowest concentration of enzyme employed (the stability of the proteins decreases when in dilute solution) V_m is essentially independent of the amount of enzyme present within the limits of the experiment.

SUMMARY

1. A study has been made of the properties of the enzyme histidase, the products formed, and the kinetics of the reaction.

2. Although the substance produced from histidine by the action of histidase has not been isolated, what is known of its chemical properties indicates that it is α -formamidino glutaric acid.

3. Histidase has been partially purified by precipitation with a concentrated potassium phosphate buffer.

4. The product was free from arginase, liver esterase, and various dehydrogenases but contained catalase and histidase.

5. On the basis of electrophoresis and phase rule studies, it is concluded that the partially purified preparation consists of a solid solution of proteins containing histidase in an unknown amount.

6. A new way of determining the constants of the Michaelis-Menten equation has been devised. The equation was applied to the histidase reaction.

7. The enzyme-substrate-complex of histidase and histidine appears to be a basic substance. It is reversibly inactivated by the addition of one hydrogen ion per enzyme molecule.

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LETTER TO THE EDITORS

The Effect of the *L. casei* Factor and of a Highly Potent Folic Acid Concentrate on Lactation in Rats Maintained on Synthetic Rations

Recent studies in this laboratory (1, 2) have shown that normal lactation is not obtained in rats or mice on highly purified diets unless the ration contains some source of "folic acid". In this communication, we wish to report the results obtained with rats on diets supplemented with the *Lactobacillus casei* factor, or with a highly potent concentrate of folic acid.

The percentage composition of the basal diet was as follows: Purified casein (Labeo or Smaco), 30; sucrose, 48; Crisco, 10; lard, 5; Osborne and Mendel's salts,¹ 5; Ruffex, 2. The vitamin supplements for this ration were as follows (per kilogram): thiamin, 20 mg.; riboflavin, 20 mg.; pyridoxin, 20 mg.; calcium pantothenate, 40 mg.; choline chloride, 500 mg.; α -tocopherol, 20 mg.; vitamin A and D concentrate, 40 mg. This is our basal diet R-5.

On this ration, the growth of rats and mice was excellent throughout four generations, and compared favorably with that of the controls kept on the stock diet. However, the lactation performance of these animals was poorer than that of the controls. According to our observations on more than 60 litters, this sub-normal lactation has shown itself in three ways: (1) a consistent loss in weight of the lactating rats, (2) the percentage of litters weaned, and (3) the average size of the litters.

The concentrate of folic acid was generously supplied by Dr. R. J. Williams. The folic acid potency of this material was 30,000. The *L. casei* factor was obtained through the courtesy of Dr. E. L. R. Stokstad, who informed us that it exhibited folic acid activity of potency 60,000. Five mg. of the folic acid concentrate was added per kilogram of diet R-5, to give diet R-17. Diet RS-5 contained 2.5 mg. of the *L. casei* factor per kilogram.

¹ The amount of MnSO_4 in the salt mixture was doubled.

Since the amounts of the folic acid concentrate and of the *L. casei* factor available to us were small, the fortified diets (diets R-17 and RS-5) were not fed until the time of mating. Previous to the feeding of the fortified diets, the rats were raised on the basal R-5 ration from the time of weaning. The results are summarized in the table below. For comparison, the data of two control experiments are given. In one of these, the rats were given diet R-5. In the other, the animals were kept on a ration containing a folic acid concentrate of potency 5000. This material was also kindly supplied by Dr. Williams, and was added to diet R-5 at a level of 25 mg. per kilogram. This is our diet R-11.

TABLE I
*Effect of a Potent Folic Acid Concentrate, and of the L. casei Factor
on Lactation in Rats.*

Diet	No. of litters	Average litter size	Average weaning weight g.	Average loss or gain weight of the mothers during lactation g.
R-5	2	4	28	-19.0
R-11	3	5	43	+ 9.0
R-17	2	8	34	-15.0
RS-5	2	7	39	+ 3.0

The data show that the diets containing the *L. casei* factor, and the folic acid concentrate had a marked beneficial effect on lactation. This effect showed itself in the larger average litter size, higher average weaning weight, and the lesser strain on the mothers during lactation. It should be noted that these results were obtained with very small quantities of the supplements.

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New York, November 1944

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Book Reviews

The Permeability of Natural Membranes. By HUGH DAVSON, Associate Professor of Physiology in Dalhousie University, and J. F. DANIELLI, Research Fellow of St. John's College at Cambridge. University Press at Cambridge, England, and Macmillan Company, New York, N. Y., 1943. x + 361 pp. Price \$4.75.

This book gives a general, but not exhaustive survey of quantitative data and their critical discussion related to the permeability of membranes, including artificial as well as natural membranes. This study is directed primarily toward an understanding of the plasma membranes of living cells and epithelia: their structure and functioning. The authors indicate that this discussion is designed for the use of professors and advanced students of biological, biochemical, and medical subjects. It will also reward careful reading by research workers in this field.

After a short introductory chapter there follow about 45 pages (Chapters II-IV) devoted to the methods used, to equilibrium conditions and equations used in permeability studies. Methods for work on erythrocytes appears to occupy a disproportionate space, some of the other work being neglected, such as the plasmometric work by Höfler. The emphasis given to the distinct functions of equilibrium conditions and rates of attainment of equilibrium is most timely and fundamental. It is unfortunate that the recent work of Conway and Boyle on muscle appeared after this chapter was written and could be given mention only. Chapter V and Appendix A treat the nature of diffusion through thin membranes—a valuable contribution since it brings out the importance of the energy content of molecules, and of temperature coefficients in permeability studies. Chapter VI discusses the modern concept of the molecular architecture of the plasma membrane, and Chapter VII the tests applicable to data in reference to current hypotheses as to the make up of the plasma membrane. There follow seven chapters (127 pages) devoted to permeability of living cells to non-electrolytes (Chapter VIII) gases, water, large molecules, ions and weak electrolytes; three to impedance and potential studies, effects of narcotics and temperature and one to hemolysis. It may be misleading that so moribund a type of cell, the erythrocyte, which deviates markedly from all other living cells should appear in this work with disproportionate emphasis. Many experiments on erythrocytes *in vivo* have been omitted. Two chapters are devoted to secretion by the intestine (resorption), the kidney and other animal and plant cells (accumulation of ions). The last chapter (XXI) treats theories of cell permeability.

The great merits of this book lie in the collation of widely scattered experimental data, and in bringing into sharp focus the outstanding questions and difficulties extant in this field. Its greatest weakness lies in the *ex parte* discussion of these aspects. Students, or even teachers lacking specialized skill or time, will be prone to accept without question the conclusions given. The authors feel that the plasma membrane consists wholly of one or more continuous bimolecular layers of lipids,

with adsorbed protein, water, etc., *i.e.* the plasma membrane is homogeneous. In Chapter VIII and Appendix A and elsewhere the authors point out that agreement within the very liberal allowance for error of 10% in one case, or five fold for the other, of data and the prediction given by them would prove the homogeneity of this membrane. This is however permissive, and not exclusive proof. Furthermore, where partition coefficients and terms involving permeability, molecular weight and temperature are correlated, especially on p. 100, the authors claim "that . . . with one possible exception . . . the points fall within the limits to be expected" (five fold) "for a homogeneous lipid membrane." But at least three out of the 16 cases in question deviate by much more, *e.g.* at least 8, and 1/9, 10 and 1/12, and 8, 1/12 and 1/47 fold. With this showing the permissive proof turns into obligate proof of the existence of an inhomogeneous membrane, a mosaic as Collander and Bärilund (the originators of the data) envisioned the plasma membrane. This agrees with the Overton "lipoid theory" of dispersed fatty materials lying in the plasma membrane. Only those previously convinced of "homogeneity" can argue as these authors do. The authors' assumption that the full area of pores in the plasma membrane is available for the free diffusion of water or solutes seems extremely misleading. Pores or spaces between molecules probably hold molecules of water and solutes, the pushing aside of these molecules requiring relatively high energies. Pores among proteins do hold water and solutes, and may be open to only a small proportion of permeant molecules, or in other words are open only part of the time. With this, the authors' dismissal of pores (pp. 102-3, 107) seems quite inadequate. Pores, conceived as openings through or between molecules, especially proteins, can fulfil the demanded requirements, and cannot be so summarily dismissed.

Furthermore there are certain defects, *e.g.* chemical potential, equated to concentration, not its logarithm (p. 1); error in assuming that volume (k , radius³) rather than area (k , radius²) determines rate of penetration (p. 41); use of permeabilities P_K and P_{Na} on both absolute and per cent bases (p. 166); wrong references to equations: 28 and 29 for 30.1 and 30.2 (pp. 215-216), 42 for 45 (p. 347), and 57 for 58 (p. 349); the reference to the "bulky" calcium ion compared with Na and K (p. 209); (the authors argue that Ca is *so large* that it cannot move as rapidly as Na or K, thus accounting for the increase of electrical resistance of tissues in $CaCl_2$ solutions, rather than Osterhout's explanation that the cells are affected by Ca; the size of the Ca ion is actually intermediate between Na and K, while it raises cell resistance while Na and K lower it); use of "e," with the same type face, in two different senses in one equation (p. 347, etc.); and so on. The reader should be constantly on guard against these. A table of the mathematical terms here used would have been particularly valuable since many are introduced without definition. Typographical errors are not rare, but only to the extent that the reader is misled as to the meaning of statements and especially of equations (*cf. e.g.* p. 341).

The writer hopes that in the future the permeability to water will be treated by using the activity of water (pure water having $a = 56.5$ moles) rather than osmotic pressure in atmospheres, as is generally done (Chapter X). The former treatment puts water on the same basis as all non-electrolytes, and facilitates reasoning from the data. The use of the present treatment of diffusion represents a real advance, but it should be remembered that its quantitative aspects are still in a preliminary state. Some changes may be anticipated.

With these reservations this book is a valuable tool; used with discretion it should help to clarify and correct many of the extant ideas, and many of the proposed ideas may well have permanent value. While unthinking use can lead only to retardation, a critical or even skeptical use must improve our understanding of the permeability of natural membranes.

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Advances in Enzymology, Volume IV, edited by F. F. NORD AND C. H. WERKMAN. Interscience Publishers, Inc., New York, 1944. 332 pp. Price \$5.50.

The issues of *Advances in Enzymology* are by now a standard work without which no biochemical library is complete. This volume is of the same high quality as the previous volumes both in selection of topics and in make-up. A new feature is the cumulative index of volumes I to IV which will be found helpful for reference work.

It is not surprising that there are no major points of criticism to be found in a collection such as this in which each chapter is written by a specialist, but it is most remarkable that the editors have been able to entice the contributors into expressing their knowledge in the terms of the average biochemist who might be reluctant to tackle a field after having tried in vain to grasp the essence of some original contributions.

The Transamination Reaction is treated by Robert M. Herbst. He discusses the model systems discovered by him as well as the enzymatic reactions concerned in a complete, well-rounded chapter. Two groups of enzymes are treated comprehensively by outstanding specialists. William Ward Pigman writes about *Specificity, Classification, and Mechanism of Action of the Glycosidases*, while J. M. Nelson and C. R. Dawson discuss *Tyrosinase*. Both contributions will assist in bringing the reader up to date on these subjects. The chapter on *Biological Energy Transformation and the Cancer Problem* by V. R. Potter is very different from most other discussions of the relation of enzymes to cancer which merely list all the attempts to find a striking peculiarity of some enzyme in cancer tissue. Potter's own interesting viewpoints make this article rather speculative at times, and some readers will disagree with him on certain points. Nevertheless there is a challenge here which will certainly stimulate others who are interested in the realm of cancer research.

H. Jensen and Leon E. Tenenbaum in their discussion of *The Influence of Hormones on Enzymatic Reactions* deplore the meagerness of our present knowledge of the mechanisms by which the hormones affect enzymatic reactions, and challenge the workers in this field to improve the situation. Facts and opinions about the effect of hormones on enzymatic reactions *in vitro* are stated briefly and concisely. This brevity contrasts pleasantly with the lengthiness employed by some investigators in this field when reporting original research of doubtful significance.

A nice new feature is the presentation of the theory and fundamentals of methods which have particularly furthered enzyme chemistry. In this volume Wallace R. Brode has undertaken the arduous task of reviewing *The Absorption Spectra of Vitamins, Hormones, and Enzymes*. Comprehensive treatment of this large field is handicapped by the limited space. The reader will find a conservative presentation of many data valuable for reference. The use of absorption spectroscopy in the study of enzyme reactions and kinetics, which has been far more important in recent

developments in this field, is barely mentioned. The reviewer hopes that the editors will again enlist the cooperation of such an outstanding specialist as Dr. Brode and will grant him more space for this side of the picture.

A valuable survey of *Gramicidin*, *Tyrocidine*, and *Tyrothricin* by Rollin D. Hotchkiss invites enzyme chemists to watch the developments in neighboring fields. It is impressive to notice how microbiologists have seized the methods of biochemistry. The most stimulating contribution to this volume is perhaps Addison Gulick's chapter on *The Chemical Formulation of Gene Structure and Gene Action*. Enzyme chemists will welcome the opportunity to become familiar with a border-line field which they hope to dominate eventually. The author devotes considerable space to explanations of elementary principles, and therefore his discussion can be enjoyed easily by the average biochemist.

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Enzyme Technology. By HENRY TAUBER. New York: John Wiley and Sons, Inc. London: Chapman and Hall, Ltd. 1943. 275 pp. \$3.50.

"Enzyme Technology" by Henry Tauber confirms many investigations that physiological changes and enzymatic action are inter-related in industry and medicine. This knowledge is furthermore enlarged by the fact that new developments of applied science are based upon old established principles. Each chapter of *Enzyme Technology* represents a highly specialized field. The first chapter on "Yeast Production and Utilization" alone covers references of over 130 authors. It is impossible in a book of such wide scope to do justice to the many different other technical processes of colloidal importance during fermentation. Qualitative principles in food industries are still the main factor. The relation of growth substances to yeast leaves many questions unanswered. For example: How much of a vitamin is needed and is there a substitute for a particular vitamin? Investigations have proven that vitamins are parts of enzyme systems which make possible the chemical changes continuously occurring in the living organism. The author does not mention new types of Yeast, Hybrids, by crossing. It is known that yeasts are quite different from most fungi since they propagate as haploid and diploid cells. In Hybridizing Yeast the structure, size, and character of the organism changes, and to a certain extent the enzymatic reaction is made more effective. In fact, these new yeast strains discovered by Winge and improved upon by Lindegren may write a new chapter in the history of Enzymes. Credit should have been given to these new researches and their possible application in the field of enzymatic changes. In Symbiosis Fermentation we have another form of stimulating enzymatic action and encouraging vitamin activity. The recognition of the value of yeast as a supplier of protein food was originated many years ago by Delbrueck. It has developed now to an industry of no mean proportion. Even small breweries send their surplus yeast to drying plants. It is against our conservation program "if a considerable amount of waste brewers' yeast finds its way into the public sewer system" (page 14). In distillation the "slop, which is usually discarded" (page 41) should have its uses in newer biological fields. Chill-proofing beers obtained with proteolytic enzymes are an advancement. But better and stable beers are still dependent upon skilful traditional methods of physiological routine in the preliminary and final stage of brewing. The author states that

"aerated fermentation results in a low protein beer of high chill resistance," and he furthermore says that "this is important in countries where proteolytic enzymes are not permitted for chill-proofing" (page 75). It would be interesting to find different principles in colloidal stabilization and pasteurization.

Enzymes in food and other industries are technically and interestingly outlined. Enzymes for medicinal use and the special chapter on Penicillin have been satisfactorily handled. It is indeed of importance that the author familiarizes investigators with the Penicillin problem. Coordination of food and fermentation research is necessary and undoubtedly plays an important role in the advancement of other sciences. The close affiliation of micro-biological function with enzyme technology should in the present and future emergency be vitally helpful for humanity everywhere.

The impression one gets is that the experience of the author is not in agreement with the variety of material presented. Besides the great gaps evidenced by the subjectivity of the treatment the reader may question in some cases the validity of the conclusions reached by the author. Thus, the statement on page 152 concerning sutures from polyvinyl alcohol is contradicted by Morrell in his recent treatise of Synthetic Resins and Allied Plastics, Oxford University Press, London, 1943, page 209.

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The Degradation of Glucose by *Aerobacter Aerogenes*

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INTRODUCTION

In 1936 it was suggested that the mechanism by which acids are formed in the mouth, with the subsequent production of dental caries, is similar to that in which alcohols and acids are formed by bacterial fermentation or that in which lactic acid is formed during muscle metabolism (1, 2, 3, 4, 5). Later (6) the postulated intermediate compounds were isolated from saliva, indicating that these reactions do occur in the mouth. In view of this situation, it is not surprising that much confusion exists concerning the type of bacteria most responsible for the production of dental caries. It is quite obvious that any micro-organism that contains or elaborates any of the enzymes necessary for the degradation of carbohydrate may play a role in the production of acids. Furthermore, when one considers the various bacteria that are present in the mouth, it is quite probable that the acids are formed by the enzymes of several bacteria growing in symbiotic relationship (7).

Insofar as the rate of acid formation in the mouth is one of the primary variables in the production of dental caries (8) it was thought interesting to study the rate of carbohydrate degradation under the influence of each of the common mouth organisms, and on the basis of this to determine the role of each type of bacteria in the production of acids in the mouth. So far these rates have been determined under the influence of *Lactobacillus acidophilus* (9), yeast (10), and *Staphylococcus albus* (11). This paper deals with *Aerobacter aerogenes*.

EXPERIMENTAL PROCEDURE

The general method used to determine the rates of various steps in the reaction was to allow a weighed amount of organisms to act for a

given period of time on each intermediate in the reaction involved in the degradation of glucose to lactic acid, and then to analyze the substrate quantitatively for the next intermediate. Each step in the procedure was performed at three pH levels: 5.4, 6.7, 8.6. The first and last values were chosen because the work of Kay (12) and others has shown that the optimum pH for phosphatase is either one or the other. The intermediate value, 6.7, was chosen because this represents the pH of normal resting saliva.

The *Aerobacter aerogenes* used in this investigation were secured from human saliva or from tooth scrapings. The organisms were then isolated in pure culture by the usual methods and grown on a solid 1% dextrose medium. Since large numbers of organisms were required, the usual methods of culturing did not readily lend themselves to the problem at hand. For this reason, a method whereby large amounts of organisms could be grown was employed (10). In all but steps II and III, the organisms were centrifuged from the harvest liquor, dried in a vacuum desiccator, and powdered finely in a mortar. In this way, the organisms could be weighed on an analytical balance, thus placing the work on a semi-quantitative basis. While the organisms prepared in this way are obviously not in their active form, the enzymes contained in them are still capable of performing their usual reactions.

I. Phosphorylation of Glucose by Aerobacter aerogenes

The method of forming and isolating hexose diphosphate and hexose monophosphate was to allow a weighed quantity of organisms to act on a known quantity of a buffered glucose solution and then determine the various intermediates by the analysis for the free phosphate, total phosphate, hexose monophosphate, and hexose diphosphate. The entire procedure was a modification of that of Harden and Henley (13), wherein the modification consisted of the addition of copper sulfate as indicated by Bauer (14), in order to slow down the dismutation of the formed hexose phosphates. Preliminary experiments indicated that the dismutation of the hexose phosphates was too rapid to permit their accumulation, so that it was necessary to conduct the reactions in .001 molar copper sulfate. This in no way interfered with the formation of the hexose phosphates. The phosphorus was determined by the method of Fiske and SubbaRow, and the glucose was estimated by the Benedict quantitative procedure. The pH was measured by a Coleman electrometer, and the hexose diphosphate isolated as the barium salt. The results of this experiment are shown in Table I.

TABLE I
Phosphorylation of Glucose by Aerobacter Aerogenes
 Concentrations in millimoles/l.

pH before incubation	5.4		6.7		8.6	
Trial	I	II	I	II	I	II
pH after incubation	5.30	5.30	6.54	6.45	8.30	7.95
Glucose before incubation	365.6	317.6	276.3	375.4	281.9	318.6
Reducing substances after	320.0	274.7	243.1	331.8	251.8	289.6
Free phosphorus before	225.2	238.7	174.2	193.5	143.2	174.2
Free phosphorus after	200.0	197.1	129.0	157.1	114.8	155.5
Phosphorus esterified	25.2	41.6	45.2	36.4	28.4	18.7
Per cent phosphorus esterified	11.2	15.9	26.0	18.8	18.6	10.7
Phosphorus esterified as diphosphate	23.3	34.1	45.2	36.4	28.4	18.1
Hexosediphosphate formed	11.7	17.1	22.6	18.2	14.2	9.1
Phosphorus esterified as monophosphate	1.9	4.0	0.0	0.0	0.0	0.6
Hexosemonophosphate formed	1.9	4.0	0.0	0.0	0.0	0.6
Per cent esterified phosphorus as diphosphate	92.7	89.6	100	100	100	96.7
Per cent esterified phosphorus as monophosphate	7.3	10.4	0.0	0.0	0.0	3.3

II. Aldolase and Isomerase in the Production of Triose Phosphates

The general methods of Utter and Werkman (15) were used in the demonstration of these two enzymes. The cell-free extract was prepared by grinding the bacteria. The resulting sludge was extracted with water and centrifuged. The clear liquid thus obtained was found to be very active.

One ml. of this enzyme preparation, 1 ml. of glycine buffer of the appropriate pH, and 1 ml. of 0.1 *M* sodium fluoride were suspended in a water bath at 37.5°. Two ml. of hexose diphosphate prepared from the calcium salt was added to start the reaction. Five ml. of 7% trichloroacetic acid were immediately added to the control tubes, containing the same mixture as described, to stop enzyme action. The tubes were incubated for two hours. At the end of this time, 5 ml. of 7% trichloroacetic acid which had been heated to the bath temperature to avoid changing the equilibrium conditions, were added to each of the reaction tubes. After centrifuging, free and total phosphorus were determined. Alkali-labile phosphate was determined as follows: an aliquot of the mixture was added to an equal volume of 2 *N* sodium hydroxide and allowed to stand for 20 minutes. It was quickly neutralized with sulfuric acid, and the free phosphate again determined.

The difference between the free phosphate before and after the alkali treatment yields the combined quantities of glyceryl aldehyde phosphate and dihydroxyacetone phosphate. The phosphate of the hexose phosphate is unaffected by this mild treatment. The iodine oxidation was used to differentiate between the glyceraldehyde phosphate and dihydroxyacetone phosphate. After the iodine oxidation, the free and labile phosphate was again determined. The phosphate of the phosphoglyceric acid is not saponified by the mild treatment. The difference between the phosphate before and after iodine oxidation gives the measure of the glyceraldehyde phosphate. The results of this experiment are found in Table II.

TABLE II
Aldolase and Isomerase in the Production of Triose Phosphates

pH	5.4		6.7		8.6	
Trial	1	2	1	2	1	2
Initial hexosediphosphate	6.50	6.50	6.50	6.50	6.50	6.50
Final hexosediphosphate	5.23	5.02	5.24	5.14	5.26	5.30
Free phosphorus after	1.77	1.85	1.75	1.91	1.85	1.86
Alkali labile phosphorus	0.72	0.85	0.73	0.67	0.61	0.56
Triosephosphate formed	0.72	0.85	0.73	0.67	0.61	0.56
Phosphoglyceraldehyde formed	0.10	0.09	0.15	0.10	0.05	0.10
Dihydroxyacetone phosphate formed	0.62	0.76	0.58	0.57	0.56	0.46
Per cent triosephosphate as dihydroxyacetone phosphate	86	89	79	85	92	82

All concentrations are in millimoles of phosphorus per liter.
Incubation time: 1 hour.

III. The Production of Phosphoglyceric Acid

The general method of Wood, Stone, and Werkman (2) was employed for this determination.

In each of several 125 cc. Erlenmeyer flasks, 7 g. of *Aerobacter aerogenes* paste, 7 ml. of 0.67 *M* phosphate buffer of the proper pH, 8 ml. of 20% glucose, 5 ml. of 2.5% sodium hexose diphosphate, and 1.5 ml. of a 0.2 *M* sodium fluoride, 0.1 ml. of toluene, and 8 ml. of sodium pyruvate, were placed, and the mixture was incubated at 37° for 24 hours. The flasks were then chilled and stored for sixteen hours in an icebox. The mixtures were centrifuged and the inorganic phosphate precipitated from the supernatant liquid by adding ammonia and treating with 20% magnesium acetate. The resulting precipitate was removed by centrifuging, and an excess of 50% barium acetate solution was added to the clear supernatant fluid. The resulting solution was stored in an icebox. At the end of 24 hours, one-ninth volume of ethyl alcohol was added to complete the precipitation. After standing in an icebox an additional 24 hours, the barium phosphoglycerate was filtered off into previously dried porous bottomed crucibles and dried and weighed. The results obtained are shown in Table III.

TABLE III

Production of Phosphoglyceric Acid by Aerobacter Aerogenes

pH	Trial	Yield of Barium Phosphoglycerate mg./100 cc.	millimoles/liter
5.4	I	284	8.84
	II	246	7.65
6.7	I	431	13.41
	II	592	18.42
8.6	I	402	12.51
	II	426	13.25

IV. The Degradation of Phosphoglyceric Acid

The degradation of phosphoglyceric acid is usually recognized by the appearance of pyruvic acid and phosphoric acid in equivalent quantities. Despite the fact that in many instances the pyruvic acid is quickly attacked and converted into other degradation products, the determination of the liberated phosphate gives a true index of the amount of phosphoglyceric acid converted. The 3-phosphoglyceric acid employed in this work was synthesized biologically by the method of Ostern and Guthke (16).

In determining the action of *Aerobacter aerogenes* on phosphoglyceric acid, the general methods of Neuberg and Kobel (17) were employed.

One gram of dried *Aerobacter aerogenes* and 1 ml. of toluene were added to 12.5 ml. of the solution of sodium phosphoglycerate. The mixture was sealed in a large test tube and shaken in a water-bath at 37.5° for 24 hours. Control tubes were prepared at each pH exactly as above, except that they contained no organisms.

At the end of the incubation period, the tubes were broken open and the contents analyzed. The proteins were precipitated with trichloroacetic acid and removed. The resulting mixture was analyzed for free phosphate, acetaldehyde, and pyruvic acid. Phosphate analyses were made by the method of Fiske and SubbaRow. Acetaldehyde and pyruvic acid were determined by the method of Simon and Neuberg (18). It might be pointed out that other carbonyl compounds in addition to acetaldehyde might also be present in the neutral precipitate. The results obtained at the various pH values are given in Table IV.

V. The Conversion of Pyruvic Acid to Lactic Acid

The general method used to determine the reduction of pyruvic acid was to permit the organisms to act with sodium pyruvate substrate in a suitable buffer solution containing a hydrogen donor. After a

TABLE IV

Action of Aerobacter Aerogenes on Phosphoglyceric Acid

pH	Trial	Phosphoric Acid Formed	Pyruvic Acid Formed	Acetaldehyde Formed
5.4	I	7.90 mmol./l.	1.81 mmol./l.	0.045 mmol./l.
	II	7.17	1.44	0.085
6.7	I	13.0	2.85	0.268
	II	13.9	3.66	0.194
8.6	I	8.34	1.76	0.138
	II	8.69	2.94	0.268

TABLE V

Action of Aerobacter Aerogenes on Pyruvic Acid

Concentrations in millimoles/100 cc.

pH before incubation	5.4	6.7	8.6
pH after incubation	5.8	6.35	6.95
Pyruvic acid before incubation	21.67	13.72	19.20
Pyruvic acid after incubation	8.29	1.93	5.21
Pyruvic acid decomposed	13.38	11.79	13.99
Per cent pyruvic acid decomposed	61.7	85.9	72.9
Acetaldehyde after incubation	5.98	6.61	7.08
Lactic acid after incubation	0.22	0.09	0.17
Per cent decomposed pyruvic as lactic	1.64	0.76	1.22
Per cent decomposed pyruvic as acetaldehyde	44.7	56.1	50.6

suitable incubation period, the mixture was analyzed for pyruvic acid, lactic acid, and acetaldehyde. In the present work, calcium glycerophosphate was used as the donor and powdered human enamel as a source of calcium phosphate.

DISCUSSION

It has been shown that *Aerobacter aerogenes* possesses the enzyme system necessary for the degradation of glucose to lactic acid.

The phosphorylation of glucose is rather rapid. In four hours *Aerobacter aerogenes* caused as much phosphorylation as *Lactobacillus acidophilus* (9) did in twenty-four hours. *Staphylococcus albus* (11) is also less active than *Aerobacter aerogenes* in this step. However, "Yeast A" (*Saccharomyces cerevisiae*) (10) esterifies two to eight times as much phosphorus at the various pH levels as *Aerobacter aerogenes* does.

The maximum amount of hexosemonophosphate was produced at the low pH value. This is in accord with the observation of Pett and

Wynne (19) that the optimum pH for phosphatase of this organism is 5.6 when hexosemonophosphate is used as the substrate.

The conversion of the hexose phosphates to the triose phosphates is not as rapid as when *Staphylococcus albus* was used. It should be noted, however, that the two are similar in that in both cases most of the triose is in the form of dihydroxy acetone phosphate.

The rate of production of phosphoglyceric acid is approximately the same as that by Yeast A at pH 5.4 and 6.7, but somewhat less at 8.6. *Aerobacter aerogenes* produces this acid considerably faster than either *Staphylococcus albus* or *Lactobacillus acidophilus*, while *Sarcina lutea* produces approximately three times as much of the acid in 24 hours as does *Aerobacter aerogenes* at the intermediate pH, but less at the low and high values.

In the degradation of phosphoglyceric acid, *Aerobacter aerogenes* is less active than *Sarcina lutea* and Yeast A, using the amount of phosphoric acid produced as the criterion. The activity of *Lactobacillus acidophilus* is about the same at pH 5.4 but much less at the two higher pH values, while that of *Staphylococcus albus* is about the same at pH 5.4 and much less at the higher levels.

Of the organisms so far studied, Yeast A is by far the most active in the aerobic production of lactic acid from pyruvic acid. *Lactobacillus acidophilus* is also quite active in this reduction, 21 to 28% of the pyruvic acid decomposed being converted to lactic acid. In the case of *Aerobacter aerogenes*, *Sarcina lutea*, and *Staphylococcus albus*, less than 2% of the decomposed pyruvic acid went to lactic acid under aerobic conditions.

SUMMARY

The degradation of glucose by the enzyme systems of *Aerobacter aerogenes* was studied. The rates at which the various intermediates were formed were determined and compared with those of the enzyme systems of other mouth organisms under similar conditions.

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The Degradation of Glucose by *Sarcina Lutea*

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INTRODUCTION

During the past few years the ability of several mouth organisms to degrade glucose to lactic acid has been studied (1, 2, 3, 4), in an attempt to determine which of the organisms may take part in the formation of lactic and other acids in the mouth with subsequent decalcification of the teeth. It is thought that when all of the known organisms that are normally present in the mouth have been studied from this point of view, more knowledge concerning the exact role of each type of organism, in relation to dental caries, will be available. This paper is concerned with the rate of glucose degradation under the influence of the enzyme systems of *Sarcina lutea*.

TABLE I
Phosphorylation of Glucose by Sarcina Lutea

pH before incubation Trial	5.40		6.60		8.85	
	I	II	I	II	I	II
pH after incubation	5.3	5.4	6.7	6.5	8.7	8.8
Glucose before incubation	0.334	0.330	0.342	0.342	0.41	0.42
Reducing substance after inc.	0.330	0.450	0.342	0.342	0.40	0.39
Free phosphorus before	0.180	0.180	0.174	0.170	0.20	0.190
Free phosphorus after	0.180	0.180	0.129	0.129	0.190	0.195
Phosphorus esterified	0.0	0.0	0.045	0.041	0.0	0.0
Phosphorus esterified as diphosphate	0.0	0.0	0.045	0.041	0.0	0.0
Hexosediphosphate formed	0.0	0.0	0.0225	0.0205	0.0	0.0
Hexosemonophosphate formed	0.0	0.0	0.0	0.0	0.0	0.0
Per cent phosphorus esterified	0.0	0.0	25.8	24.1	0.0	0.0
Per cent phosphorus esterified as diphosphate	0.0	0.0	100.0	100.0	0.0	0.0
Per cent phosphorus esterified as monophosphate	0.0	0.0	0.0	0.0	0.0	0.0

TABLE II
Aldolase and Isomerase
millimoles/liter

pH	5.4	6.7	8.9
Initial hexosediphosphate	5.85	5.85	5.85
Final hexosediphosphate	5.75	5.63	5.56
Free phosphorus after inc.	0.07	0.00	0.05
Alkali labile phosphorus	0.13	0.44	0.58
Triosephosphate formed	0.13	0.44	0.58
Phosphoglyceraldehyde formed	0.00	0.07	0.10
Dihydroxyacetone phosphate formed	0.13	0.37	0.48
Per cent triosephosphate as dihydroxyacetone phosphate	100.0	84.10	82.80

TABLE III
Production of Phosphoglyceric Acid by Sarcina Lutea

pH	Trial	Yield of Barium Phosphoglycerate mg./100 cc.	mmol./liter
5.4	1	157.5	4.93
5.4	2	170.0	5.33
6.6	1	1490	46.6
6.6	2	1250	39.0
8.4	1	46	1.44
8.5	2	40	1.25

TABLE IV
Action of Sarcina Lutea on Phosphoglyceric Acid

pH	Trial	H ₃ PO ₄ mmol./l.	Pyruvic Acid Formed, mmol./l.	Acetaldehyde Formed, mmol./l.
5.6	1	12.11	5.95	0.18
	2	9.45	4.32	0.12
6.6	1	36.02	46.70	2.73
	2	28.62	40.40	3.75
8.6	1	18.27	10.32	0.22
	2	15.44	8.46	0.36

The organisms were isolated and grown as previously described and all of the experiments were conducted in exactly the same manner as described in the preceding paper. The results are shown in Tables I to V inclusive.

TABLE V
Action of Sarcina Lutea on Pyruvic Acid
 Concentration millimoles per liter

	Without Hydrogen Donor			With Glycerophosphate			With Hexosediphosphate		
pH before	5.4	6.6	8.4	5.4	6.7	8.5	5.5	6.6	8.4
pH after	6.2	6.0	7.1	6.4	6.5	6.8	6.6	6.6	6.8
Pyruvic acid before	160.0	160.0	160.0	160.0	160.0	160.0	160.0	160.0	160.0
Pyruvic acid after	8.3	4.2	10.2	21.3	7.5	18.2	12.0	4.5	7.8
Pyruvic acid lost	151.7	155.8	149.8	138.7	152.5	141.8	148.0	155.5	152.2
Lactic acid formed	1.066	2.32	1.89	1.74	2.77	2.40	1.02	1.35	1.42
Acetaldehyde formed	2.22	2.86	2.52	7.83	8.50	10.12	2.1	0.80	1.3
Per cent pyruvic acid decomposed	94.7	97.5	93.51	86.5	94.7	88.5	92.5	97.0	95.0
Per cent decomposed acid (pyruvic) converted to lactic acid	0.71	11.50	1.26	1.25	1.82	1.69	0.69	0.87	0.93

EXPERIMENTAL

The Formation of Hexose Phosphates

The enzymes of this organism did not form hexose phosphate at the higher and lower pH values, but did form the diphosphate at pH 6.6. Only the diphosphate was formed. This is in contrast with yeast, *Lactobacillus acidophilus*, *Aerobacter aerogenes* and *B. subtilis*, as in each of these cases some hexose monophosphate was formed. The rate of reaction at pH 6.6 was greater than that of *Lactob. acidophilus* and *Staph. albus* at their optimum pH values, but slower than yeast and *A. aerogenes*.

Aldolase and Isomerase

The conversion of the hexose phosphates to the triose phosphates is not as rapid as when *Staph. albus* or *A. aerogenes* was used. It should be noted that at pH 5.4 all of the hexose phosphate was converted to dihydroxy acetone phosphate. At the other pH values, a small portion was converted to glyceraldehyde phosphate. *Staph. albus* and *A. aerogenes* also cause these reactions to go primarily to the ketone.

The Production of Phosphoglyceric Acid

Sarcina lutea also produced phosphoglyceric acid most abundantly at pH 6.7. It also produced a small amount at pH 5.4 and 8.5. This organism is by far the most active of any of the above mentioned organisms in respect to the rate of phosphoglyceric acid production. It would thus be expected that if the enzymes of all of the above organisms were present and could act simultaneously, the yeast would form the hexose phosphate rapidly and the *Sarcina lutea* would rapidly utilize it, thus causing the over-all reaction to proceed very rapidly, providing the proper pH was available.

The Formation of Pyruvic Acid

This organism was also quite active in producing pyruvic acid at pH 6.6, although yeast was more active at a different pH. Considerable amounts of pyruvic acid were isolated at this stage of the reaction when *Lactob. acidophilus*, *A. aerogenes*, and yeast were used. This is in contrast with the results when *Staph. albus* was used. In this case the pyruvic acid was destroyed as fast as it was formed.

The Formation of Lactic Acid

Very little lactic acid was formed by the action of *Sarcina lutea* on pyruvic acid. Here again the most active pH was 6.6. At this reaction practically all of the pyruvic acid was decomposed, but the major end product was acetic acid. The reaction went rapidly, and, unlike the other organisms, did not require a hydrogen donor.

CONCLUSIONS

On the basis of these results, *Sarcina lutea* has the ability to degrade glucose to lactic acid. The first reaction is quite slow, but the second and third are very rapid. The end product is primarily acetic acid rather than lactic acid. On the basis of these results, *Sarcina lutea* may be able to carry the third reaction faster than any of the organisms so far tried.

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Observations on the Occurrence of "Folic Acid" in Liver and Muscle

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INTRODUCTION

Wright and Welch observed (1, 2) that when rat liver was digested with xanthopterin more "folic acid" was found in the tissue than when the digestions were carried out in the absence of added pterin. Also, when fresh rat liver was incubated with several natural materials, more "folic acid" appeared in the digestion mixtures than could be accounted for by separate assays of the constituents. These observations have been confirmed by Totter, Mims, and Day (3).

This paper is concerned with additional observations on the occurrence of "folic acid" in liver and muscle.

PROCEDURE

"Folic acid" was determined by the Landy and Dicken method (4) in which *Lactobacillus casei* is employed as the test organism. Occasional additional determinations have been made by the Mitchell and Snell method (5) with *Streptococcus fecalis* R. A crude "folic acid" concentrate served as the working standard. This standard in turn was assayed against a crystalline "folic acid" isolated from liver and the results are reported in terms of the presumably pure material.

Samples of xanthopterin employed included material prepared by the methods of Purrmann (6), Koschara (7), and Totter (8). All samples showed similar activity.

The taka-diastase used was a commercial product. The enzyme preparation in the amounts used did not introduce a significant "folic acid" blank.

All digestions were carried out in stoppered flasks at 37°C. One ml. of benzene was added to each flask to minimize bacterial contamination. Since the composition of the digestion mixtures was not constant, a description of each procedure used is given in the table or figure in which the results obtained are reported. After enzymatic digestion all samples were autoclaved at 15 pounds pressure for 10 minutes

and the homogenized tissue diluted to such volume that 1 ml. was equivalent to 25 mg. of the fresh weight of the tissue sample. Suspended material was removed by suction filtration. Appropriate dilutions of the filtrates were used in the microbiological assays.

TABLE I

"Folic Acid" Content of Digested Rat Liver As Influenced by Various Conditions of Dispersion, pH, and Taka-Diastase

Experiment No.	Liver sample No.	Method of dispersing tissue	Digestion medium	Taka-diastase	"Folic acid" observed
				per cent	γ per g.
1	1-9	Spatula	Water		4.0(2.2- 5.4)
	1-9	"	"	5	9.6(5.0-13.6)
2	10	"	"		7.2
	10	"	"	2	11.4
3	11	"	pH 7.0 buffer		3.6
	11	"	" 7.0 "	2	3.6
	11	Waring blender	" 7.0 "		2.4
	11	" "	" 7.0 "	2	2.2
4	12	Spatula	" 7.0 "		3.6
	12	"	" 7.0 "	2	4.0
	12	Waring blender	" 7.0 "		2.8
	12	" "	" 7.0 "	2	2.6
5	13	" "	" 7.0 "		4.0
	13	" "	" 7.0 "	2	3.8
	13	" "	" 7.0 "	5	3.6
	13	" "	" 7.0 "	10	3.8
	13	" "	" 7.0 "	20	4.4
6	14	" "	" 4.0 "		1.4
	14	" "	" 4.0 "	2	1.2
	14	" "	" 7.0 "		2.6
	14	" "	" 7.0 "	2	2.0

In the experiments involving dispersion of the liver with a spatula, approximately 2 g. of liver were ground up in 20 ml. of water or 20 ml. of 0.05 *M* phosphate buffer of pH 7.0. In the experiments involving liver mixed in the blender, the liver was ground in either 0.1 *M* phosphate buffer of pH 7.0 (2 g. of liver per 10 ml. of buffer) and diluted with an equal volume of water, or, as in Experiment 6, ground in water (2 g. per 10 ml.) and diluted with an equal volume of 1% acetate buffer of pH 4.0. Incubation was for 24 hours at 37°C.

RESULTS

The effect of various incubation procedures on the observed "folic acid" content of rat liver and skeletal muscle is described below.

Variations in pH, State of Dispersion, and Presence of Taka-Diastase. When taka-diastase was employed, a greater yield of "folic acid" was obtained from rat liver fragmented in water than from hepatic tissue allowed to autolyze in water (Table I). If the water was replaced with

TABLE II

"Folic Acid" Content of Digested Rat Muscle As Influenced by Various Conditions of Dispersion, pH, Taka-Diastase, and Xanthopterin

Experiment No.	Muscle sample	Method of dispersing tissue	Digestion medium	Taka-diastase	Xanthopterin	"Folic acid" observed
				per cent	γ	γ per g.
1	1	Waring blender	pH 4.0 buffer			0.08
	1	" "	" 4.0 "		200	0.08
	1	" "	" 4.0 "	2		0.22
	1	" "	" 4.0 "	2	200	0.21
	1	" "	" 7.0 "			0.027
	1	" "	" 7.0 "		200	0.027
	1	" "	" 7.0 "	2		0.14
	1	" "	" 7.0 "	2	200	0.11
2	2	" "	" 4.0 "			0.030
	2	" "	" 4.0 "		200	0.031
	2	" "	" 4.0 "	2		0.078
	2	" "	" 4.0 "	2	200	0.094
	2	Spatula	Water			0.023
	2	" "	" "		200	0.032
	2	" "	" "	2		0.059
	2	" "	" "	2	200	0.059
3	3	Waring blender	"	2		0.13
	3	" "	pH 7.0 buffer	2		0.12
	3	" "	" 4.0 "	2		0.15

In the experiments involving dispersion of the muscle with a spatula, approximately 2 g. of muscle were ground up in 20 ml. of water. In the experiments involving muscle mixed in the blender, the muscle was ground in water (2 g. of muscle per 10 ml. of water) and diluted with an equal volume of either 1% acetate buffer of pH 4.0 or 0.1 M phosphate buffer of pH 7.0, as indicated. Incubation was for 24 hours at 37°C.

sodium phosphate buffer of pH 7, the amount of "folic acid" was less and the yield was not increased by taka-diastrase. When rat liver that had been mixed in a Waring blender was digested in acetate buffer of pH 4, as recommended by Cheldelin *et al.* (9), the "folic acid" content did not increase on addition of taka-diastrase.

Experiments with rat skeletal muscle gave results quite different from those obtained with rat liver (Table II). Muscle that had been fragmented or mixed in a blender gave considerably higher results after digestion at pH 4 than at pH 7. In addition the effect of taka-diastrase in liberating bound "folic acid" from combination in muscle was readily demonstrated at pH 4.

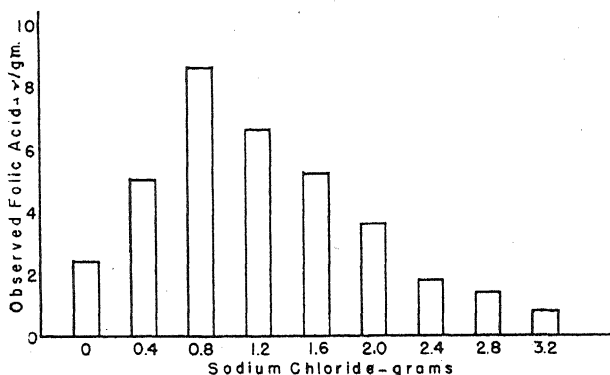


FIG. 1

The Observed "Folic Acid" Content of Rat Liver after Incubation with Varying Amounts of Sodium Chloride

The indicated amounts of sodium chloride dissolved in 10 ml. of water. 10 ml. portions of 20% blenderized rat liver in pH 7.0 *M*/10 phosphate buffer, containing taka-diastrase equivalent to 2% of the weight of the liver used, added to each flask. Incubated for 24 hours at 37°C.

Incubation with Neutral Salts. Incubation of rat liver, but not of rat muscle, with moderate amounts of neutral salts increased the amount of "folic acid" in subsequent microbiological assays (Figs. 1 and 3). Incubation of both rat liver and muscle with large amounts of sodium chloride caused a decrease in the "folic acid" content. It was demonstrated that the amount of salt used did not affect the growth of *Lactobacillus casei*.

Varying Amounts of Xanthopterin. Maximal values for the "folic acid" content of the tissue (Fig. 2) were obtained on incubation of rat liver with an amount of xanthopterin approximately equivalent to 50 times that of the "folic acid" present. Digestion with either minute amounts of xanthopterin or relatively large amounts of neutral salts caused a definite increase in the "folic acid" content of rat liver that had been mixed in a blender. In the presence of suboptimal amounts of sodium chloride xanthopterin brought about a further increase in

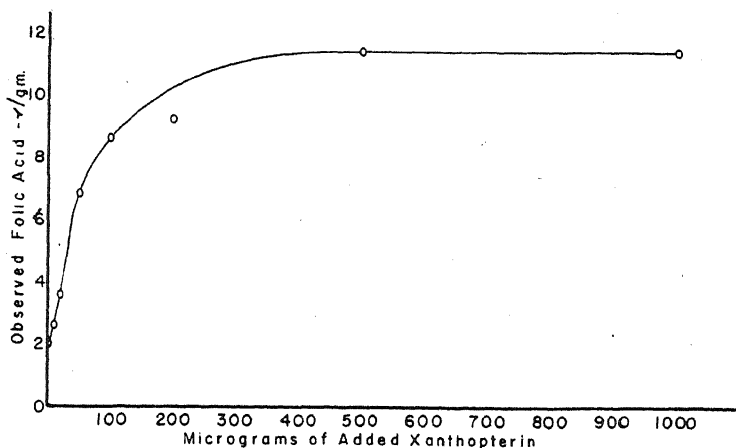


FIG. 2

The Observed "Folic Acid" Content of Rat Liver after Incubation with Varying Amounts of Xanthopterin

The indicated amounts of xanthopterin present in 10 ml. water. 10 ml. portions of 20% blenderized rat liver in pH 7.0 M/10 phosphate buffer, containing taka-diastase equivalent to 2% of the weight of the liver used, added to each flask. Incubated for 24 hours at 37°C.

the yield of "folic acid" but the effects of these two materials were not entirely additive. There appeared to be a maximal "folic acid" value which could not be increased by the addition of any amount of sodium chloride or xanthopterin singly or in combination (Fig. 3).

Variations in pH. A definite maximum in the "folic acid" content of rat liver was found after incubation at about pH 8 (Fig. 4). In the presence of xanthopterin the maximum was found at a slightly lower pH, but the effect of xanthopterin was also evident in more acid solu-

tions. In these experiments the buffered solutions were equivalent in their content of inorganic phosphate.

Duration of Incubation and Various Combinations of Taka-Diastase, Sodium Chloride, and Xanthopterin. The apparent "folic acid" content of identical series of mixtures, containing only rat liver that had been

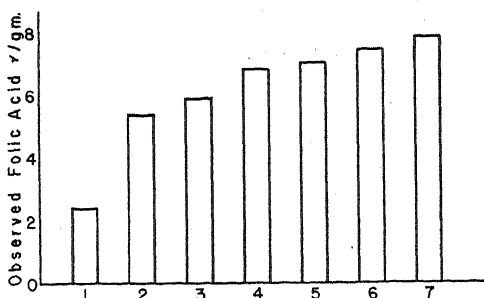


FIG. 3

The Observed "Folic Acid" Content of Rat Liver after Incubation with Varying Amounts of Neutral Salts and Xanthopterin

The indicated supplements dissolved in 10 ml. of water. 10 ml. portions of 20% blenderized rat liver in pH 7.0 *M*/10 phosphate buffer, containing taka-diastase equivalent to 2% of the weight of the liver used, added to each flask. Incubated for 24 hours at 37°C.

- No. 1 No supplement
- No. 2 200 γ xanthopterin
- No. 3 1.2 g. Na_2SO_4
- No. 4 1.2 g. Na_2SO_4 plus 200 γ xanthopterin
- No. 5 0.5 g. NaCl
- No. 6 0.5 g. NaCl plus 200 γ xanthopterin
- No. 7 2.4 g. Na_2SO_4

mixed in a blender or liver plus taka-diastase, sodium chloride, or xanthopterin, alone or in combination, that were incubated at pH 7 for 2, 18, or 72 hours, is shown in Fig. 5. The highest "folic acid" values were obtained after 72 hours of incubation in the presence of a combination of sodium chloride, taka-diastase, and xanthopterin.

In another experiment several series of mixtures were prepared and assayed after varying periods of incubation (Fig. 6). As incubation progressed, the "folic acid" content of the samples containing xanthopterin rose to levels significantly higher than were found in the absence

of added pterin. The recoverable "folic acid" increased slightly at first and then decreased to a low level in the samples that contained only liver or liver plus taka-diastase.

In a third type of experiment, not reported in detail, a higher "folic acid" content was demonstrated in the presence of xanthopterin only

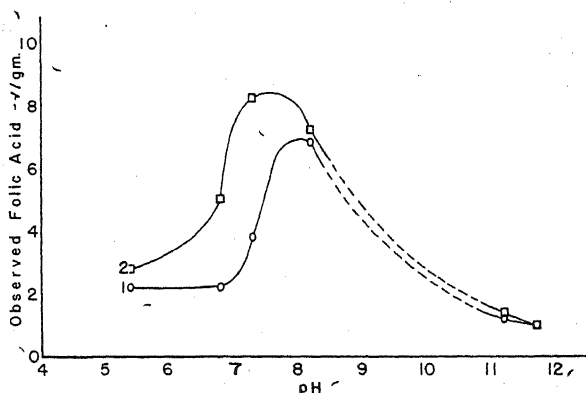


FIG. 4

The Observed "Folic Acid" Content of Rat Liver after Incubation at Various pH Values Alone and in the Presence of Xanthopterin

M/5 NaH_2PO_4 , Na_2HPO_4 , and Na_3PO_4 mixed to give 10 ml. volumes of buffered phosphate of the pHs indicated. 2 ml. water added to the series containing liver alone: (1) 2 ml. of a solution of 100 γ /ml. of xanthopterin added to the supplemented series; (2) 10 ml. portions of 20% blenderized rat liver in water, containing taka-diastase equivalent to 2% of the weight of the liver used, added to each flask. Incubated for 24 hours at 37°C.

when the pterin was present from the beginning of the incubation period.

Method of Dispersion. In three successive experiments the liver from a single rat was divided into portions and treated in several ways. The effect of taka-diastase in liberating "bound folic acid" was observed when the liver was incubated in water in a fragmented condition (liver alone, average 4.4 γ per g.; liver plus taka-diastase, average 6.8 γ per g.). Under these conditions xanthopterin had little effect in increasing the amount of "folic acid" in the tissue (average 7.2 γ per g.). However, no effect of taka-diastase was discernible with liver that was mixed in a blender with a buffer of pH 7 and the levels of

"folic acid" were lower than when incubation took place in water (liver alone, average 2.5 γ per g.; liver plus taka-diastase, average 2.0 γ per g.). Xanthopterin under these conditions caused a several fold increase in the amount of "folic acid" (average 7.0 γ per g.), but the amount of "folic acid" found when liver was fragmented in water in the absence of xanthopterin was of the same order (6.8 γ per g.).

Addition of Cyanide. Regardless of the presence of xanthopterin only small amounts of "folic acid" were found when incubations of

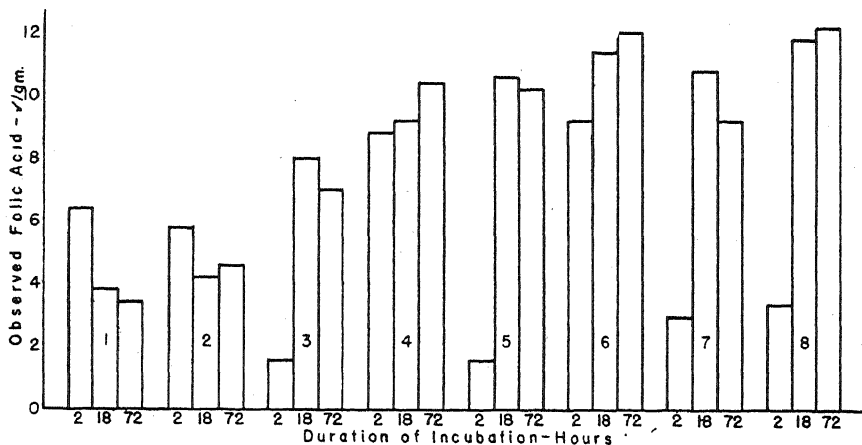


FIG. 5

The Observed "Folic Acid" Content of Rat Liver after Incubation for Varying Lengths of Time Alone and in the Presence of Taka-Diastase, Sodium Chloride, and Xanthopterin Singly and in All Possible Combinations

The individual supplements dissolved in 10 ml. of water. 10 ml. portions of 20% blenderized rat liver in pH 7.0 M/10 phosphate buffer added to each flask.

- No. 1 Liver alone
- No. 2 Liver plus taka-diastase
- No. 3 Liver plus 0.8 g. sodium chloride
- No. 4 Liver plus xanthopterin
- No. 5 Liver plus taka-diastase plus sodium chloride
- No. 6 Liver plus taka-diastase plus xanthopterin
- No. 7 Liver plus sodium chloride plus xanthopterin
- No. 8 Liver plus sodium chloride plus taka-diastase plus xanthopterin

Where taka-diastase was employed in the digestion flasks it was present in an amount equivalent to 2% of the weight of the liver used. Where xanthopterin was employed it was present in the amount of 200 γ per flask.

liver were carried out in the presence of cyanide (Table III). Separate experiments have shown that the amount of cyanide used in the incubations was not sufficient to influence the results of the subsequent microbiological assay of the liver samples.

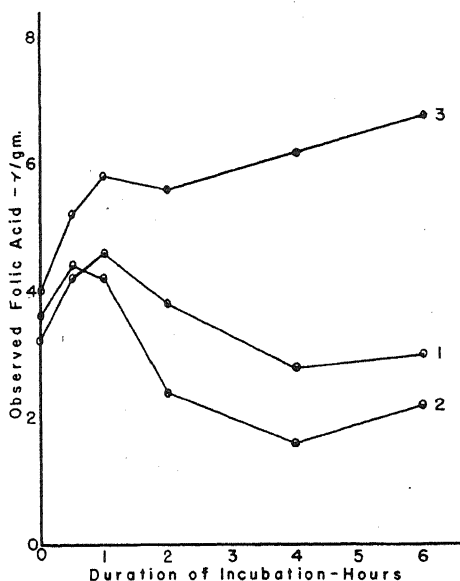


FIG. 6

The Observed "Folic Acid" Content of Rat Liver after Digestion for Varying Lengths of Time Alone or in the Presence of Taka-Diastase or Taka-Diastase and Xanthopterin

The indicated amounts of xanthopterin or xanthopterin plus taka-diastase prepared in 10 ml. of water. 10 ml. portions of 20% blenderized rat liver in pH 7.0 M/10 phosphate buffer added to each flask. Incubated for periods of time as shown.

No. 1 Liver alone

No. 2 Liver plus 2% taka-diastase

No. 3 Liver plus 2% taka-diastase plus 200 γ xanthopterin

DISCUSSION

"Folic acid" apparently functions in at least two reactions which occur during the digestion of rat liver at 37°C. One reaction involves the release of the growth factor from naturally occurring complexes. The second reaction involves a change in "folic acid" to a form with

TABLE III

"Folic Acid" Content of Rat Liver after Incubation with Sodium Cyanide
and with Cyanide and Xanthopterin

Supplement to digested liver	"Folic acid" observed γ per g.
None	2.6
1 mg. sodium cyanide	1.4
5 " " "	1.2
10 " " "	1.0
200 γ xanthopterin	9.0
200 " " + 1 mg. sodium cyanide	4.4
200 " " + 5 " " "	3.0
200 " " + 10 " " "	2.0

The indicated supplements were dissolved in 10 ml. of water. To each flask were added 10 ml. portions of 20% rat liver mixed in a blender with 0.1 M phosphate buffer of pH 7.0, containing taka-diestase equivalent to 2% of the weight of the liver used. Incubation was for 24 hours at 37°C.

considerably less or no activity as a growth factor for *Lactobacillus casei* or *Streptococcus fecalis* R. The data indicate that this inactivation takes place most readily at acid reaction and when the hepatic tissue is highly dispersed. It will be recalled that excretion studies in man have shown (1) that only a very small proportion of ingested "folic acid" appears in the urine.

The effect of xanthopterin in increasing the amount of "folic acid" after incubation with rat liver is consistent with the hypothesis that the action of the pterin is due to its probable structural similarity to "folic acid," which enables it to interfere with the metabolism of the growth factor.

The increase in the "folic acid" content of liver tissue incubated in the presence of sodium chloride may also be interpreted as dependent upon interference with the inactivation of "folic acid." The reaction in which "folic acid" is liberated from bound forms apparently is retarded by relatively large amounts of sodium chloride.

This study has been limited to a consideration of "folic acid" in rat liver and muscle. Such information would appear fundamental to an understanding of the role of rat liver in the production of "folic acid" from natural materials *in vitro* (1).

SUMMARY

Experiments are described which demonstrate that the "folic acid" content of rat liver and muscle may be materially influenced during a

preliminary digestion process by such factors as the presence of "digestive enzymes," by pH, by the degree of dispersion of the tissue, by duration of the digestion, and by the presence of neutral salts, xanthopterin, or cyanide.

The data obtained are interpreted as indicating that in the liver of the rat "folic acid" may undergo enzymatic conversion into materials having little or no activity as growth factors for lactic acid organisms. No evidence indicating the disappearance of "folic acid" during the incubation of rat skeletal muscle could be found.

ACKNOWLEDGMENTS

We wish to express our thanks to the Lederle Laboratories for a sample of crystalline "folic acid" from liver. Dr. A. H. Land and Dr. J. M. Sprague of these laboratories and Dr. J. R. Totter of the University of Arkansas supplied the xanthopterin used in these experiments.

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The Determination of Iron in Tissues ¹

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INTRODUCTION

The red color of the iron-thiocyanate complex has long been used as a basis for standard methods of iron analysis.

A comparison of the molar extinction coefficients of iron complexes as obtained on the Beckman spectrophotometer and from certain published spectrophotometric data (Table I) indicates that the thiocyanate method is more sensitive than others that have been described. Because of high sensitivity and the strong acidity of the colored solution, the thiocyanate method is particularly suitable for biological application.

The methods for total iron described below are essentially those of the Association of Official Agricultural Chemists (1) and of Kennedy (7) adapted to small amounts of iron and to the Beckman spectrophotometer. An application of thiocyanate to direct determination of iron in tissue extracts is presented in method "c."

a. Semi-micro method for total iron: 0.5 ml. each of 60% HClO_4 and concentrated H_2SO_4 are added to not over 0.5 g. tissue in a 15 cm. glass stoppered test tube and the whole digested to dryness. The salts are dissolved in 2.5 ml. 5% HNO_3 by boiling for 15 seconds. 1.0 ml. concentrated HCl , 5.0 ml. water and 2.0 ml. 20% KSCN are added. Immediately, optical density readings from 540 to 450 $\text{m}\mu$ at 10 $\text{m}\mu$ intervals are made with the Beckman spectrophotometer. These readings divided by the appropriate factors (see Table II) give the amount of iron in the sample in micrograms. The following are essential characteristics of the method:

Range 0 to 70 γ

Precision 0.2 γ

0.1 ml. of blood or 0.2 g. of liver are suitable samples for analysis.

b. Micro-method for total Fe: Digestion is carried out as above. The salts are dissolved in 1.0 ml. 5% HNO_3 plus 0.1 ml. concentrated HCl by boiling for 15 seconds. 5.0 ml. water and 2.0 ml. 20% KSCN are added and the tube cooled below

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TABLE I
Molar Extinction Coefficients of Iron Complexes

Complex	Solvent	Wave length ($m\mu$)	Mol. ext. coef.	Published data		
				Wave length ($m\mu$)	Mol. ext. ¹ coef.	References
Thiocyanate	60% acetone	480	16,000	(ca. 485) ²	14,000	(20)
	Iso-amyl alc.	490	12,500	490	13,200	(7)
<i>o</i> -Phenanthroline	H ₂ O	480	10,000	(ca. 475) ²	7,000	(20)
	H ₂ O	510	11,000	490-510	9,900-11,500	(4, 5, 12, 15, 20)
α , α' -Bipyridyl	H ₂ O			510-522	8,370-8,650	(4, 9, 15, 20)
Thioglycolic acid	H ₂ O	540	4,140	535	3,760-3,910	(8, 18, 20)
Terpyridyl	H ₂ O			552	11,500	(15)
Nitro- <i>o</i> -phenanthroline	H ₂ O			490-505	11,130	(12)
					11,410	
Disodium-1,2-dihydroxybenzene-3,5-disulfonate	H ₂ O			480	6,300	(21)
	(pH = 9.4)					
	H ₂ O			620	1,700	(21)
	(pH = 4.0)					
8-Hydroxyquinoline	CHCl ₃			470	6,000	(13)
Salicylaldoxime	H ₂ O			480	2,580, 4,940	(6, 20)
Kojic acid	H ₂ O			400	4,250	(14)
Ferron	H ₂ O			610	3,740-3,840	(17, 20)
Salicylic acid	H ₂ O			520	1,622-1,665	(10, 11, 20)

¹ Calculated in most cases from data published in terms of per cent transmission.

² "Wave length of maximum absorption."

room temperature. 3.5 ml. isoamyl alcohol are added and the tubes shaken.² The iso-amyl alcohol layer should remain clear. Readings are taken on the extract from 540 to 450 $m\mu$ and divided by the factors in Table II to give the amount of iron in micrograms. The characteristics of the method are:

Range 0 to 15 γ

Precision 0.05 γ

0.01 ml. blood, 0.5 ml. blood serum, or 0.1 g. muscle are suitable examples.

² An alternate method adapted from Woods and Mellon (20) is more sensitive but may not be as satisfactory. The ash is dissolved in 0.7 ml. 5% HNO₃ and 0.3 ml. concentrated HCl, 2.0 ml. acetone and 0.5 ml. 20% KCNS are added and the color determined spectrophotometrically.

c. Free iron of tissue extracts: The tissue is dispersed in a glass grinder to form a fine suspension if necessary and made up to 6.0 ml. with water. 0.1 ml. concentrated HCl is added and the suspension extracted three times with 5 ml. portions of ether to remove hematin. Emulsions are broken by centrifuging at 5000 r.p.m. in an angle centrifuge. To 5.0 ml. of the extracted suspension is added 2.0 ml. 20% KSCN and 3.5 ml. isoamyl alcohol. After shaking, the mixture is centrifuged. If

TABLE II
Factors for Calculation of Iron
(L = 1 cm.; Conc. = 1 γ)

Wave length $m\mu$	Semi-micro Total Fe	Log I_0/I Micro Total Fe	Free Fe of tissues
540	0.01094	0.0470	0.0342
530	0.01250	0.0527	0.0381
520	0.01400	0.0573	0.0414
510	0.01520	0.0611	0.0442
500	0.01615	0.0634	0.0459
490	0.01686	0.0641	0.0466
480	0.01701	0.0626	0.0450
470	0.01659	0.0590	0.0423
460	0.01589	0.0529	0.0379
450	0.01490	0.0459	0.0329

the alcohol becomes cloudy in the absorption cells, it is warmed gently. Readings are made from 540 to 450 $m\mu$ and the amount of iron calculated from the factors in Table II. The useful range is from 0 to 20 γ iron, but the precision is not better than $\pm 5\%$ for reasons given below. 0.025 ml. blood cells, 0.5 ml. blood serum, or 0.2 g. liver are suitable samples.

The absorption spectrum of an isoamyl alcohol extract prepared in this manner in a typical case (Fig. 1) shows the presence of a small amount of hematin. By measuring the absorption from 410 to 380 $m\mu$ and dividing by the factors in Table III, the apparent concentration of hematin in arbitrary units can be calculated.

TABLE III
Factors for Calculation of Hematin
(L = 1 cm.; Conc.—arbitrary)

Wave length $m\mu$	Log I_0/I
410	0.249
400	0.310
390	0.298
380	0.275

The ratio:

$$R = \frac{\text{Hematin calc. from 410 to 380 } m\mu}{\text{Fe calc. from 540 to 450 } m\mu}$$

is a linear function of the ratio $\frac{\text{True Fe}}{\text{Apparent Fe}}$. The percentage of the apparent iron concentration that is true iron can be calculated from the equation:

$$\% \text{ Fe} = \frac{1 - R}{0.009625} \cdot 3$$

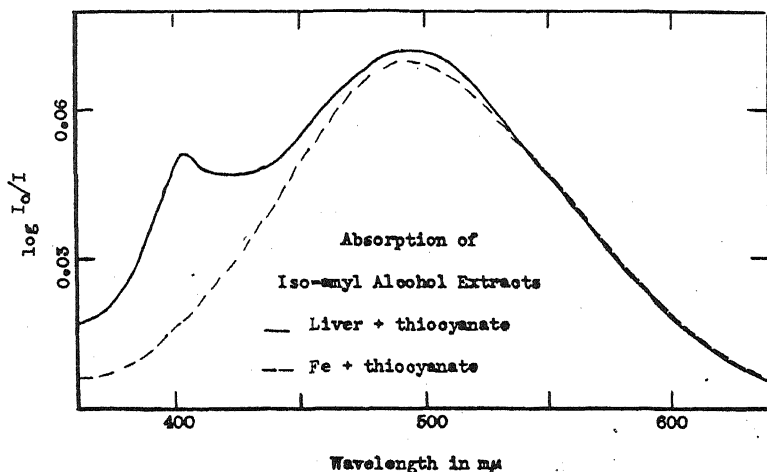


FIG. 1

“Free” iron of iron compounds and complexes: The percentage of total iron of various iron compounds and complexes which reacts with thiocyanate is shown in Table IV. The ferritin, isolated from horse

³ The hematin extinction coefficients in Table III, determined with pure hematin, were so calculated that when no Fe was present, $R_{500} = 1.000$. When no hematin was present R became accordingly equal to 0.0375. If, in the equation

$$\% \text{ Fe} = \frac{1 - R}{0.009625}, \quad \% \text{ Fe} = \frac{C_{\text{Fe}} \times 100}{\frac{E_{500}^{\text{Fe}}}{E_{500}^{\text{Hem}}} + E_{500}^{\text{Hem}}} \bigg/ \frac{E_{500}^{\text{Fe}}}{\epsilon_{500}^{\text{Fe}}},$$

R is given its value of

$$\frac{\frac{E_{400}^{\text{Fe}} + E_{400}^{\text{Hem}}}{\epsilon_{400}^{\text{Hem}}}}{\frac{E_{500}^{\text{Fe}} + E_{500}^{\text{Hem}}}{\epsilon_{500}^{\text{Fe}}}},$$

$\epsilon_{500}^{\text{Hem}}$ is made equal to $\epsilon_{500}^{\text{Fe}}$, and proper substitution is made for extinction in terms of C and ϵ , the constant 0.009625 will be found equal to $\frac{\epsilon_{500}^{\text{Hem}} - \epsilon_{400}^{\text{Fe}}/\epsilon_{400}^{\text{Hem}}}{\epsilon_{400}^{\text{Fe}}/\epsilon_{400}^{\text{Hem}}} \times 100$. (ϵ = extinction coefficient, c = true concentration, E = extinction.) Although only two wave lengths are considered here for simplicity, the equation is equally valid as used in practice, and is the simplest method of calculation for two-color systems when more than two wave lengths are considered.

TABLE IV
Percentage of Total Iron that Reacts as Free Iron

Type of iron compound	Model	Per cent
Simple Ion	Fe^{+2} , Fe^{+3}	100
Simple Complex	Phosphate, Citrate	100
	Pyrophosphate	
Iron-Protein Complex	Fe^{+3} + Gelatin	91
Iron Oxide	FeOOH sol + Gelatin (dialyzed)	10
	Precipitated FeOOH sol. (dialyzed)	2
	Ferritin	23
	Hematin (from hemin)	0
Iron-porphyrin	Rat Hemoglobin (cryst.)	6

spleen and dialyzed, was donated by Dr. L. Michaelis. The hemoglobin was crystallized from water and recrystallized twice from 20% ethanol.

DISCUSSION

Difficulties have often been encountered in the thiocyanate method for iron. These have been minimized in the methods here presented by use of good grade thiocyanate (Mallinckrodt A.R.) and proper acidity and because few interfering ions are present in animal tissue ash. In the presence of light, the thiocyanate in water solution in the first method decomposes in a few hours to colloidal sulfur and to HCN. This does not occur in the other two methods. Although calculations would be simplified by measuring absorption at only one or two wave lengths, the accuracy of these methods is increased by the use of several readings. Further, the presence of contaminating colors is readily indicated by non-uniformity of the concentration calculated at different wave lengths. This may be of particular importance in the free iron method.

Primary causes of error in these methods are contamination and inability to obtain a uniform, accurately measured sample. Contamination can largely be eliminated by the use of a minimum of apparatus. Under well-controlled conditions these sources of error are much smaller than the individual variations of animal tissue iron content.

The method for free iron of tissues here presented measures a fraction of iron that is similar to that of certain earlier methods (2, 3, 16,

19). The exact origin or significance of the iron fraction measured by any of these methods is not clear. It has been found, however, that our method is useful in determining the forms in which iron is present in tissues.

In the absence of strong reducing agents, both ferrous and ferric iron are determined in the free iron method because the equilibrium between these two forms is shifted by removal of ferric iron. The accuracy of the method is limited because, after ether extraction, the insoluble portion of the tissue suspension gathers at the interface and occludes a certain amount of ether. The error introduced by the presence of the ether is about 5%.

SUMMARY

Methods have been developed for the spectrophotometric determination of iron in animal tissues with thiocyanate.

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The Respiratory Metabolism of Ram Spermatozoa *

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INTRODUCTION

The metabolism of ejaculated ram spermatozoa has been investigated by a number of workers, Comstock (1), Chang and Walton (2), C. F. Winchester and McKenzie (3, 4), Moore and Mayer (5).

Moore and Mayer (5) studied glycolysis of ram spermatozoa in semen and found that when the lactic acid produced was neutralized at intervals (to prevent the harmful effect of acid), motility continued for 20 hours or more after all sugar had been glycolytically removed. Their results indicated that the ram sperm possesses an energy reserve other than carbohydrate and a mechanism for utilizing this reserve material in the maintenance of motility. In bull spermatozoa it has been found that, in the absence of glycolyzable sugars, the oxidative utilization of the phospholipid reserve furnishes the energy for motility (6, 7).

Chang and Walton (2) have studied the respiration of ram spermatozoa in diluted seminal fluid and have demonstrated the adverse effect of temperature shock.

No investigation has heretofore been reported of the metabolism of ram spermatozoa washed free of seminal fluid, or of the ability of these cells to utilize metabolites other than glucose. Such studies are reported in this paper.

EXPERIMENTAL AND RESULTS

The semen used in these studies was collected from healthy rams by means of an artificial vagina. The time between collection of the specimens and initiation of the manometric measurements varied usually from one to two hours but was found not to influence the rate of respiration of normal specimens, as will be shown. The technique

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of preparing sperm suspensions free of seminal fluid has been described elsewhere (8). The respiration experiments were carried out as previously described (8) except that micro Warburg flasks having a total volume of 7 to 8 cc. were used and the final volume of the sperm suspension in each flask was one cc. Motility observations were made on all specimens following the manometric experiments (9).

Phospholipids were extracted by the method of Bloor (10), and the phosphorus determined colorimetrically after wet ashing with H_2SO_4 and HNO_3 .

In studying the effect of ionic composition of the suspension fluid on the metabolism of ram spermatozoa, it was found that a modified Ringer-phosphate medium, previously found suitable for bull spermatozoa (8) was likewise suitable for studying ram spermatozoa. Calcium, a constituent of the suspension media used by many investigators, is detrimental, even in extremely low concentrations, to ram spermatozoa. A concentration of 0.0018 M CaCl_2 in the suspension medium caused a mean decrease in respiration of 10% in 3 experiments. This level of calcium caused no detectable effect on motility. A concentration of 0.0044 M inhibited respiration 65%, depressed motility and caused clumping of the spermatozoa.

Effect of pH on Respiration

While we can confirm the observations of C. F. Winchester and McKenzie (4) that the rate of respiration of ram spermatozoa falls off under extremely acid or alkaline conditions we found pH not to influence respiration of *washed* ram spermatozoa over the narrow range of pH 6.5 to 7.5. In a series of 4 experiments, each with semen from a different ram, variations in pH of the buffer medium from 6.5 to 7.5 resulted in no significant variations in respiration. In each of these experiments media of pH 6.5, 6.8, 7.0, 7.25, 7.4, 7.5 were used. Since motility seemed to be retained better near pH 7.25, buffers of this pH were used throughout the remainder of the work herein reported. This is the pH which Winchester and McKenzie (4) report as optimum for respiration of ram spermatozoa in diluted semen.

Effect of Sperm Concentration on Respiration Rate

In the case of bull spermatozoa it was reported (7) that the respiration rate was independent of sperm concentration in suspensions con-

taining from 0.1 to 1 billion spermatozoa per 3 cc. Using ram and boar semen, C. F. Winchester and McKenzie (3) found decreased respiration with higher concentrations of spermatozoa.¹ In the present studies it was found that the rate of respiration of washed ram spermatozoa was independent of concentration where the sperm concentration was from 1 to 5.0×10^8 per cc. This was found to hold for the endogenous respiration of the spermatozoa from each of 4 individual rams tested and was likewise found to hold fairly closely for the respiration in the presence of glucose, pyruvate and succinate as shown in Table I.

TABLE I
Effect of Concentration on Respiration Rate

Spermatozoa/cc.	Endogenous	Glucose	Zo ₂	Pyruvate	Succinate
1.1×10^8	-29.6	-37.0		-45.3	-37.2
2.2×10^8	-34.5	-34.5		-42.7	-33.8
3.9×10^8	-33.5	-33.5		-40.5	-38.4

Zo₂ = c.mm. O₂/10⁸ sperm cells/hour. Temperature, 37°C.

Effect of "Age" on Respiration

It has been previously shown that when bull semen was stored for varying periods of time, the subsequent rate of respiration varied inversely with the time of storage (7). The Zo₂ values of specimens held four hours were about one half those of fresh specimens. In the present work it was found that the initial rate of respiration of ram spermatozoa was retained much longer. Specimens of vigorous ram spermatozoa could be washed free of seminal fluid and stored up to seven hours at room temperature without affecting the subsequent rate of respiration at 37°C. as shown in Table II.

During this period of storage at room temperature the spermatozoa retain an excellent degree of motility. Since washing the spermatozoa

¹ The decrease in respiration with increasing sperm concentration observed by C. F. Winchester and McKenzie (3) is undoubtedly the result of limiting oxygen diffusion into the liquid phase. With the high sperm concentrations employed by these workers the suspensions are very viscous (especially in the case of boar semen) and even rapid shaking of the flasks causes little agitation of the surface. Their argument that oxygen diffusion could not have been limiting because yeast cells gave linear respiration is not valid for their data (Fig. 4, page 653) show that the total oxygen consumption of the yeast was far below that of either ram or boar spermatozoa.

with isotonic buffer almost completely removes the glucose contained in the original semen, the spermatozoa probably utilize their lipid reserve as a source of energy during this time. The ram sperm must either contain a larger reserve of phospholipid, or utilize it more efficiently than does the bull sperm, for the spermatozoa of the ram maintain the initial high degree of motility for a much longer time

TABLE II

Effect of "Age" of Ram Spermatozoa on Respiration

Time between collection and beginning of measurement	Z _O ₂				
	Endogenous	Egg Phospholipid	Glucose	Pyruvate	Succinate
1 hr. 17 min.	-27.1	-33.8	-25.6	-33.7	-30.9
2 hrs. 36 min.	-31.9	-30.9	-25.4	-35.0	-31.9
4 hrs. 4 min.	-28.1	-31.6	-23.0		
6 hrs. 49 min.	-28.5		-21.9		

1.5 cc. of semen from Ram No. 134 diluted to 7.5 cc. with buffer solution, centrifuged, the fluid discarded and the spermatozoa suspended in 15.5 cc. fresh buffer solution. Stored at room temperature. 0.9 cc. of this suspension was used per flask. Sperm count 2.9×10^8 /flask. Temperature 37°C.

than do bull spermatozoa. Ether-alcohol extractable phospholipids of ram spermatozoa were therefore determined for comparison with the phospholipid content of bull spermatozoa. Washed spermatozoa from three different rams contained 9.0, 9.0, and 9.2 μ g. lipid P/ 10^8 cells as compared with 6.2, 6.6, 7.4, and 7.7 μ g. lipid P/ 10^8 cells for four normal specimens of bull spermatozoa. It thus appears that the ram sperm actually does have a somewhat greater reserve of phospholipids.

Effect of Various Metabolites on Respiration

In Table III are shown the rates of endogenous respiration, and the effect of added metabolites on the oxygen consumption, of ejaculates of ram spermatozoa collected at varying intervals through the breeding season. The average endogenous Z_O₂ for 20 ejaculates (including those in Table III) was 22.4 which agrees with previous preliminary results (8). While the high rate of endogenous respiration of the ram sperm largely obscures the effect of added metabolites, certain of the substances tested did appreciably affect the oxygen consumption and some of them definitely prolonged or improved the motility of the sperm during the manometric experiments.

TABLE III

Effect of Various Metabolites on the Respiration of Ram Spermatozoa

Ram	Endoge- nous	Glucose	Pyru- vate	^{ZO₂} Phospho- lipids	Succi- nate	Malate
G	-10.5	-29.0				
G	-17.0	-19.9	-28.1	-19.0	-23.1	
G	-15.1	-17.2				
1024	-32.5	-35.0	-42.8		-36.5	
1046	-25.1	-46.0	-66.0		-38.9	-31.4
8102	-13.1	-15.1	-22.3		-13.6	
134	-11.9	-11.0	-13.6		-21.0	-12.6
134	-28.1	-25.8	-28.4		-32.0	
134	-16.8	-20.9	-30.9	-27.4	-23.5	
134	-27.1	-25.6	-33.7	-33.8	-30.9	
121	-22.8	-15.4	-20.0		-20.0	
121	- 8.5	-11.0	-14.1			
322	-19.1	-18.0	-23.1		-24.0	

Experiments carried out at 37°. ZO_2 values recorded are the average of duplicates. Phospholipid added at 5 to 10 mg./cc., all other substrates were present in a final concentration of 0.02 M.

Egg phospholipids (7) increased respiration² only slightly but seemed to be beneficial in prolonging motility. Glucose did not consistently depress the respiration of rapidly metabolizing ram sperm (as it does in the case of bull sperm), and it even caused considerable increases in the respiration of spermatozoa from certain ejaculates.

Succinate increased respiration appreciably and improved maintenance of motility. In other experiments citrate (either with or without further additions of magnesium) did not influence respiration but, as in the case of bull spermatozoa (8), it was found to be beneficial to motility.

Pyruvate additions to ram spermatozoa suspensions caused the greatest increases in respiration, but the oxidation of this metabolite did not always seem to be coupled with energy utilization. For example, in the specimen from ram No. 1046 pyruvate more than doubled the respiration without any improvement in motility after two hours at 37°. Both succinate and malate gave much smaller increases in respiration but definitely improved motility. Similar observations were made on the ejaculates from rams No. 1024 and 8102.

² All ZO_2 values in the presence of phospholipid are corrected for the slight autooxidation of the phospholipid preparation.

These seem to be exceptional cases for at least two lines of evidence indicate that pyruvate can furnish energy for maintenance of motility. First, in all those cases where pyruvate caused only a comparatively small increase in respiration, there was a definite improvement in maintenance of motility. Secondly, as shown in Table IV, pyruvate

TABLE IV
Metabolite Utilization in the Presence of DNP

Substrate addition	Control		0.0001 M DNP	
	ZO ₂	Motility*	ZO ₂	Motility*
None	17	3+	15	1+
Glucose	21	4+	32	2+
Pyruvate	29	4+	37	2+
Succinate	24	4+	28	1+
Egg Phospholipid	27	4+	27	2+

* Observed after the manometric measurement of respiration (2 hours at 37°). The data are the average of 2 similar experiments.

supports motility in the presence of 2,4-dinitrophenol—a reagent which inhibits the utilization of the lipid reserve for the maintenance of motility (11).

The indication that egg phospholipid may benefit motility in dinitrophenol-treated ram spermatozoa is interesting in view of the fact that it is not beneficial to DNP-treated bull spermatozoa.

DISCUSSION

While the metabolic behavior of the ram sperm is, in general, very much like that of the spermatozoa of other species of domestic animals, it does possess certain distinctive features. Outstanding is the high degree of "vigor" as displayed by its (1) higher rate of respiration, (2) its greater store of endogenous metabolites, (3) its ability to maintain its initial rate of metabolism for long periods of time, (4) its excellent maintenance of motility during storage. Both the endogenous respiration (8) and the respiration in the presence of added metabolites have been found to be greater for the ram sperm than for the sperm of other species thus far investigated. It is well known that ram spermatozoa retain motility during storage longer than spermatozoa from other domestic animals.

The ram sperm also differs from that of the bull in its ability to utilize four-carbon dicarboxylic acids such as succinate and malate as

sources of energy for maintenance of motility. Normal bull spermatozoa appear to be impermeable to these acids while both normal and dinitrophenol-treated ram spermatozoa readily oxidize them.

The independent ability of either glycolytic or oxidative processes to furnish the energy for motility of ram spermatozoa has been demonstrated by the same procedures employed for studying this in bull spermatozoa (6, 12). For example, cyanide inhibits both respiration and motility in the absence of glucose. When glycolyzable sugars are provided, however, cyanide does not inhibit motility.

The considerable variation in respiration rates of the spermatozoa of different ejaculates from the various rams may have been the result of collecting semen at irregular intervals or of insufficient training of the rams to serve the artificial vagina.

SUMMARY

An investigation of the oxygen consumption and motility of ejaculated ram spermatozoa washed free of the constituents of the seminal fluid has been made. Considerable variation in the rate of respiration of the spermatozoa was found between different rams and also between various ejaculates of the same ram.

The rate of oxygen consumption, by spermatozoa of any given ejaculate, was found to be constant with varying sperm concentrations in the range 1.0 to 5.0×10^8 cells/cc. Maximum respiration was obtained with suspension media of pH 6.5 to 7.5 while motility was best retained in a medium of pH 7.25.

Ram spermatozoa retain their original rate of respiration and motility even when washed free of glucose and other seminal fluid constituents and stored for several hours. Analyses indicate that ram spermatozoa have a somewhat higher content of phospholipid than do bull spermatozoa.

Additions of pyruvate, succinate, or egg phospholipids increased respiration and prolonged motility. Spermatozoa from certain ejaculates oxidize pyruvate at an extremely rapid rate. Motility in these specimens was not improved by the presence of pyruvate indicating that this abnormally rapid oxidation was not coupled with energy utilization.

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The Metabolism of Bovine Epididymal Spermatozoa *

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INTRODUCTION

The metabolism of mammalian epididymal spermatozoa has been studied by a number of investigators: Wolf (1), Redenz (2), Windstösser (3), Henle and Zittle (4, 5), but until the recent investigation of Henle and Zittle (4) it was not known that the epididymal sperm possesses metabolic characteristics far different from those of the ejaculated sperm. The metabolic characteristics in which epididymal spermatozoa differ from ejaculated spermatozoa are, notably, (a) failure to show a constant rate of respiration with sperm suspensions of varying density, (b) the necessity of a substance found in epididymal secretion (4, 6) to stabilize their respiration so that a constant rate can be observed, (c) lower endogenous respiration, and (d) stimulation of the respiration by glucose in contrast to the depression of endogenous respiration observed when glucose is added to ejaculated spermatozoa.

It seems likely that these metabolic differences may be related to the reported (7) differences in fertilizing capacity between the "immature" epididymal and the "mature" ejaculated spermatozoa.

EXPERIMENTAL AND RESULTS

Bovine epididymides, obtained at a local packing plant, were brought to the laboratory about one hour after the bulls were slaughtered. They were kept in the refrigerator until used for experimentation. The spermatozoa were removed by injecting physiological saline into the distal end of the vas deferens and draining the spermatozoa from an incision in the epididymis as described by W. Henle (8). The saline suspension was centrifuged, the supernatant discarded, and the sperma-

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tozoa taken up in calcium-free Ringer-phosphate (9), pH 7.4. Other experimental details were essentially as described elsewhere (9) except that micro Warburg vessels (total volume 7 to 8 cc.) were used and the final volume of the sperm suspensions in the flask was one cc. Motility observations were made on all specimens after the manometric measurements and the degree of motility recorded as 1+ to 5+ as previously described (10).

Phosphate partition studies were made by precipitation of the sperm proteins with cold trichloroacetic acid, adjusting the pH of an aliquot of the filtrate to 8.2, and precipitation of the inorganic phosphate and esters insoluble in CaCl_2 solutions with 1/5 volume of 10% CaCl_2 solution saturated with calcium hydroxide. The calcium salts were dissolved with a minimum of acid, and aliquots were analyzed for inorganic phosphorus by the method of Fiske and Subbarow (11) before and after hydrolysis in 1 N HCl at 100° for various periods of time.

With the exception of sorbose and gentiobiose, the sugars used were the purest samples available commercially. *l*-Sorbose was prepared by bacterial oxidation of *d*-sorbitol with *Acetobacter suboxydans*.¹ Following treatment of the fermentation media with norite and filter cell the sorbose was isolated and purified according to Bates and associates (12). β -Octa-acetyl-gentiobiose was synthesized by the method of Reynolds and Evans (13). It was deacetylated by the catalytic barium methylate procedure of Isbell (14), and the gentiobiose was crystallized as the di-methyl alcoholate. Before experimental use, the methyl alcohol was removed by vacuum distillation of an aqueous solution of the crystalline sugar alcoholate.

Lactic acid was determined by the method of Barker and Summerson (15).

Initiation of Motility and Phosphate Uptake

Upon removal of the spermatozoa from the epididymis, they were found to be almost completely immotile. Within a few minutes, however, motility began to increase rapidly. The initiation of motility was not accelerated by additions to the sperm suspension of succinate, oxalacetate, glucose, phosphate, calcium or potassium chloride, sodium bicarbonate or acetyl choline, but did seem to be accelerated by agitation and aeration of the sperm suspension. There is at present no evidence of a specific activating agent responsible for the initiation of motility, but an important factor in the maintenance of motility of extruded spermatozoa, in contrast to the quiescence of the spermatozoa within the vas deferens, is the access to a sufficient oxygen tension to initiate oxidative processes which, through coupling with phosphorylation, can furnish the energy for motility (16, 17). When aerobic metabolite oxidation was prevented by the addition of cyanide (0.001 M) to the sperm suspension as soon as possible after removal from the epididymis, motility failed to improve above the 1+ (<10%

¹ We wish to thank Mr. D. Perlman for carrying out the bacterial oxidation.

of the sperm showing progressive motility) rating which was present at the time cyanide was added.

We had previously found (16) that ejaculated bull spermatozoa contain appreciable quantities of an acid labile ester which appeared to be adenosine triphosphate.² The concentration of this ester decreased during storage and had completely disappeared when the spermatozoa were no longer motile. When glucose or phospholipids were added, the original level of adenosine triphosphate was maintained for a longer time, and motility likewise was retained. In order

TABLE I
Phosphorylation in Epididymal Spermatozoa

Treatment	Motility at 20 min.	"A.T.P."	Δ Inorganic. phosphorus during incubation
		μg. P/10 ⁶ cells	μg. P/10 ⁶ cells
Initial control *		17	
Incubated control	3+	25	- 9
+ 0.02 M glucose	4+	25	-11
+ 0.004 M cyanide	<1+	4	+17
+ 0.02 M glucose + 0.004 M cyanide	3+	21	- 9

The data are the average of two similar experiments. In each experiment the spermatozoa were rapidly removed from 2 epididymides and made up to the desired volume with saline-phosphate.

* This initial control sample was immediately pipetted into cold trichloroacetic acid. The other samples were incubated 20 min. at room temperature, aerobically.

further to clarify the role of this labile phosphate ester in the initiation of motility, studies were made of the changes in phosphate partition in the spermatozoa immediately following removal from the epididymis. The results are shown in Table I.

The initial control sample was precipitated as soon as a suspension of uniform density could be obtained. The time required for removal

² The rate of hydrolysis of the phosphoric acid esters insoluble in CaCl₂ solution indicated that the easily-hydrolyzable fraction probably was adenosine triphosphate phosphorus. Hexose-diphosphate, the other ester insoluble in CaCl₂ solution which is usually present in this fraction, and which would be partially hydrolyzed during the 7 min. hydrolysis, was absent from the calcium salts of sperm filtrate. There was no further liberation of inorganic phosphorus after 7 min. hydrolysis in 1 N HCl at 100°.

of the spermatozoa from the epididymis, mixing with saline-phosphate buffer and precipitation of the initial control aliquot was about one minute. It is possible that part, but probably not likely that all, of the "A.T.P." found in the initial control sample was synthesized during this time, for precautions were taken to keep aeration at a minimum. The data show that *the oxidation of the phospholipid stores*^{3,4} *results in an uptake of inorganic phosphorus, an increase in phosphate esters insoluble in calcium salt solutions and hydrolyzable in 7 min., and the maintenance of motility.* When the oxidation was inhibited by cyanide (18), the "A.T.P." decreased with an equivalent increase in inorganic phosphorus. In the presence of glucose, cyanide did not inhibit motility, "A.T.P." levels were increased and inorganic phosphorus taken up. In the spermatozoa then, both the aerobic oxidation of lipids and the anaerobic glycolysis of glucose is coupled with phosphate esterification. Similar conclusions regarding the ability of two separate processes to furnish energy for motility were previously reached using various metabolites under aerobic and anaerobic conditions (19) and by selective inhibition (18).

Rates of Respiration and Glycolysis

In confirmation of the observations of Henle and Zittle (4), it was found that the rate of respiration of epididymal bull spermatozoa increased with increasing sperm concentration to the point where solubility of oxygen in the suspension medium became the limiting factor. As shown by Henle and Zittle (4) a constant rate of oxygen consumption can be obtained when epididymal secretion is added to the medium. To facilitate comparisons of results from various experiments, all of the experiments reported in this paper were performed using sperm suspensions with a density of 2.0 to 3.0×10^8 cells/flask. In any given experiment a constant number of spermatozoa were used in all flasks.

The rates of respiration and glycolysis of fresh epididymal spermatozoa in the modified Ringer-phosphate medium, found suitable for studies of ejaculated bull spermatozoa (9), are shown in Table II. The rates of respiration agree with, but those of glycolysis in the calcium-free Ringer-phosphate are lower than, those reported by Henle and Zittle (4), who employed a medium containing a higher potassium

³ The possibility of a carbohydrate metabolism is eliminated by the following facts. Freshly removed spermatozoa contain a negligible amount of glucose (reducing substance) and under anaerobic conditions, no detectable production of lactic acid occurs unless sugars are added.

⁴ Epididymal bull spermatozoa contain about the same amount of ether-alcohol extractable phospholipids [average of four samples = $6.4 \mu\text{g. lipid E}/10^9$ cells] as do ejaculated spermatozoa [average of four samples = $7.0 \mu\text{g. lipid P}/10^9$ cells (20)].

TABLE II

Respiration and Glycolysis Rates of Epididymal Bull Spermatozoa

Number of experiments	Z_{O_2}		Z_L	
	Endogenous	Glucose	Air	N ₂
	13		19	5
Range	-3.1 to -10.2	-6.2 to -15.5	7.4 to 16.4	42 to 67
Average	-6.5 (± 1.9)	-10.4 (± 3.5)	12.7 (± 2.5)	56 (± 9.6)

Spermatozoa for each experiment were pooled from four epididymides, each from a different bull. Final concentration of added glucose (in last three columns) was 0.01 *M*. The suspension medium was calcium-free Ringer-phosphate (9), pH 7.4. Temperature, 37°C. Z_{O_2} = c.mm. $O_2/10^8$ cells/hour. Z_L = lactic acid produced (equivalent to c.mm. $CO_2/10^8$ cells/hour).

concentration and, in addition, 0.025 *M* bicarbonate and an unusually high $CaCl_2$ concentration (0.019 *M*). When this concentration of bicarbonate was added to our medium, higher rates of glycolysis were also obtained.

Effect of Calcium and Other Ions. In view of the previously observed high toxicity of calcium to ejaculated bull (9) and ram (20) spermatozoa, and the high rate of respiration and glycolysis reported by Henle and Zittle for epididymal bull spermatozoa in a medium containing calcium, the effect of various calcium levels was investigated. The results are shown in Table III. Calcium at all concentrations tested proved detrimental to respiration, glycolysis and motility. When added at a level of 0.019 *M*, respiration was negligible and both glycolysis and motility were absent.

TABLE III

Effect of Calcium on Metabolism of Epididymal Bull Spermatozoa

Calcium salt concentration	Z_{O_2}		Z_L^{air}	Motility at 1 hr.
	Endogenous	Glucose*		
<i>M</i>				
0.000	-8.2	-13.6	12.5	4+
0.0025	-8.0	- 6.9	7.6	2-3+
0.005	-6.7	- 5.9	6.3	2+
0.010	-5.3	- 5.5	4.0	1+
0.019	-2.0	- 1.0	<0.1	0

* Glucose, when added, was present in a final concentration of 0.02 *M*. Temperature, 37°C.

In view of the excellent respiration and motility reported by Henle and Zittle, who routinely employed this high concentration of calcium, experiments were carried out to determine the influence of other constituents of their suspension medium on the effect of calcium on the spermatozoa.⁵ The results are shown in Table IV. Increasing the potassium content of the medium (to the level employed by Henle and Zittle) was found not to alter the effect of calcium. Likewise, bicarbonate (which Henle and Zittle employed in their glycolysis experiments) did not prevent the

TABLE IV

Effect of Various Salts on Metabolism of Epididymal Bull Spermatozoa

Molarity of salts in medium			Z_{O_2}		Z_L^{air}	Motility at 1 hr.
KCl	CaCl ₂	NaHCO ₃	Endogenous	Glucose		
0.005			-6.5	-8.8	7.4	4+
0.035			-8.9	-5.4	10.8	4+
0.005	0.019		-2.0	0.0	1.6	F.M.**
0.035	0.019			0.0	1.5	V.F.M.*
0.005		0.005			33.0	5+
0.035		0.005			27.0	5+
0.035	0.019	0.005			1.3	V.F.M.
0.005	0.019	0.025			4.8	V.F.M.

** F.M. = Few motile.

* V.F.M. = Very few motile.

deleterious effects of calcium on glycolysis and respiration. Bicarbonate did, however, stimulate glycolysis in the calcium-free medium, and when a concentration of 0.025 *M* was used the rates of glycolysis were consistently brought to the same high level reported by Henle and Zittle (4).

Utilization of Added Metabolites

Redenz (2) reported that lactate and pyruvate increased respiration, but not motility, of epididymal sperm suspensions containing no sugar. We had found pyruvate and acetate to increase respiration, and support motility, of epididymal bull spermatozoa (21). As shown in Table V, intermediates in carbohydrate and fat metabolism as well as several of the intermediates of the "Krebs' cycle" increased both the oxygen consumption and motility. The sperm utilizes the lactic acid of *l* configuration only. *trans*-Aconitic acid, and propionic and butyric acids were not utilized. Studies on the mechanism of oxidation

⁵ These experiments were carried out at the suggestion of Dr. C. A. Zittle to whom our results were privately communicated.

TABLE V

Effect of Various Metabolites on the Respiration of Epididymal Bull Spermatozoa

Metabolite	Number of experiments	% Increase above endogenous respiration	Motility after 2 hrs.
Endogenous	6	†	2+
Glucose	5	58	4+
Pyruvate	4	157	4+
<i>l</i> -Lactate*	4	133	4+
<i>d</i> -Lactate**	1	0	2+
Succinate	2	33	4+
Malate	2	8	3+
Citrate	3	19	3+
Isocitrate	2	16	3+
<i>cis</i> -Aconitate	2	25	3+
<i>trans</i> -Aconitate	3	0	2+
Acetate	3	26	4+
Propionate	1	4	1+
Butyrate	1	17	1+
Acetoacetate	1	75	4+
β -Hydroxybutyrate	3	-10	4+
Egg Phospholipids	3	35	5+

† Endogenous ZO_2 averaged 9.0 in these 6 experiments.* Sodium salt of *l*-lactic acid.** Sodium salt of *d*-lactic acid. We are indebted to Dr. M. J. Johnson for samples of these pure optical antipods.

of these various metabolites have been made and are presented in another paper (22).

Sugar Utilization. The ability of epididymal spermatozoa to form lactic acid from various sugars is shown in Table VI. An interesting observation was made regarding the comparative ability of epididymal

TABLE VI

Utilization of Various Sugars by Epididymal Spermatozoa

Sugar	Z_L^{air}	$Z_L^{N_2}$
Glucose	12.9	60
Fructose	13.8	62
Mannose	10.0	67
Maltose	3.0	3.8
Others tried*	<0.1	<0.1

* Galactose, *l*-sorbose, *d*-ribose, sucrose, cellobiose, gentiobiose. All sugars were added to 0.01 *M*. Temperature, 37°C.

and ejaculated spermatozoa to utilize maltose. With epididymal sperm, maltose was utilized at an extremely slow rate, and was slightly beneficial in supporting motility. In view of the previous finding that ejaculated spermatozoa can glycolyze maltose as rapidly as glucose (23), the same sugar solutions used in the experiments reported in Table VI were again tested with ejaculated spermatozoa. Glycolysis of maltose was found to be slightly more rapid than glucose. It is therefore unlikely that the maltose contained any glycolytic inhibitory substance, and it seems established that epididymal spermatozoa cannot glycolyze maltose nearly as rapidly as they can glucose, fructose, or mannose. The ability of the mammalian sperm to utilize gentiobiose was investigated because of the report of Kuhn, *et al.* (24) that gentiobiose is extremely active as an aerobic motility factor for the gametes of the green algae, *Chlamydomonas eugametos*, and that crocin, the naturally occurring gentiobiose glycoside of crocetin, induces motility in the absence of oxygen. Neither epididymal nor ejaculated spermatozoa could produce lactic acid from gentiobiose, and it was without beneficial effect on motility.

TABLE VII
*Effect of Storage of Excised Epididymides on the Respiration of
Their Contained Spermatozoa*

Trial	Time after excision of epididymides					
	3 hours		24 hours		48 hours	
	Z_{O_2}					
	Endogenous	Glucose	Endogenous	Glucose	Endogenous	Glucose
A	-6.5	-11.7	-13.4	-14.0		
B	-8.2	-13.6	-10.2	-13.2	-13.8	
C	-3.7	- 6.4	- 6.3	- 8.9	-14.0	-11.6
D	-6.0	-15.5			-11.7	-15.6

The same sperm concentration was employed in all experiments in any given trial. Temperature, 37°C.

Increased Endogenous Respiration in Stored Spermatozoa

During the course of these investigations it was observed that when whole epididymides were stored in the refrigerator one or two days before removing the

spermatozoa, the endogenous respiration of the spermatozoa was appreciably greater than for fresh specimens. Respiration in the presence of glucose remained essentially unchanged. In Table VII, each trial represents one lot of epididymides of which four were taken at random for each day's experiment. Although the validity of such results is limited by the variation in respiration rate of spermatozoa from different epididymides (4), the consistency with which an increased endogenous respiration was observed indicates that the increase is real and not the result of experimental variations. This increase in endogenous respiration to a level approaching that in the presence of glucose may be related to the normal "maturation" processes of the sperm in the intact epididymis, and may be caused by the accumulation by the sperm of the constituents of epididymal secretion (4, 6) which are beneficial to epididymal spermatozoa.

DISCUSSION

From the results obtained in this study, several interesting comparisons may be made of the metabolism of epididymal spermatozoa with those obtained from semen. Under similar conditions of investigation, the rates of respiration and aerobic glycolysis of epididymal spermatozoa are lower, while anaerobic glycolysis is higher, than those of the ejaculated sperm. In the presence of bicarbonate, however, the rates of glycolysis are increased above those obtained with ejaculated spermatozoa. In the presence of dinitrophenol aerobic glycolysis is increased to the level of anaerobic glycolysis. Dinitrophenol did not increase anaerobic glycolysis but was detrimental to motility.

The rates of the various metabolic processes observed in this investigation agree with those obtained by Henle and Zittle (4), but we were unable to confirm their rates of respiration or glycolysis when using suspension media containing the same calcium concentration as they employed. This amount of calcium (0.019 *M*) always inhibited respiration, glycolysis and motility. Glucose increased the respiration of epididymal bull and (in other experiments) rabbit spermatozoa.

The present finding that lipid oxidation in spermatozoa is coupled with phosphate esterification and the production of an acid-labile ester which appears to be adenosine triphosphate is the first clear demonstration that fat oxidation is coupled with phosphorylation. In the case of the endogenous respiration of spermatozoa, it has been shown that the substance utilized is phospholipid (23, 19), and, furthermore, carbohydrate is absent.³ The function of the adenosine triphosphatase in the sperm (17) may be related to the utilization of the energy-rich phosphate esters for the maintenance of motility. Incidentally, the concentration of "A.T.P." in spermatozoa (one μ g. easily hydrolyzable P/mg. dry weight) is about the same as that in striated muscle.

Epididymal spermatozoa readily utilize a variety of intermediary metabolites including pyruvate and lactate, four carbon dicarboxylic acids, the naturally occurring tricarboxylic acids, and acetate, acetoacetate, and β -hydroxybutyrate. Each of these prolongs maintenance of motility and usually causes an appreciable increase in oxygen consumption. β -Hydroxybutyrate was unusual in that it depressed respiration slightly but supported an excellent degree of motility. It is possible that the oxidation of this metabolite is, in spermatozoa, more efficiently coupled with phosphorylation than is the oxidation of the endogenous lipid reserve. The utilization of these intermediates will be discussed in the following paper (22).

SUMMARY

The metabolism of bovine epididymal spermatozoa was studied and the following results obtained.

Initiation of motility in bull spermatozoa after removal from the epididymis was hastened by aeration and could be prevented by addition of cyanide to the medium. Freshly removed epididymal spermatozoa incubated aerobically esterified inorganic phosphate to produce an ester which appeared to be adenosine triphosphate. *Both the oxidation of the endogenous lipid stores and the glycolysis of glucose are independently coupled with this esterification.*

The rates of respiration and aerobic glycolysis of epididymal are much lower than those of ejaculated bull spermatozoa. The rate of anaerobic glycolysis is higher, and both aerobic and anaerobic glycolysis are greatly stimulated by bicarbonate.

Bovine epididymal spermatozoa utilize pyruvate, L-lactate, a number of intermediates of Krebs' isocitric acid cycle, acetate, acetoacetate, β -hydroxybutyrate, and egg phospholipids under aerobic conditions for the maintenance of motility.

Epididymal bull spermatozoa (like the ejaculated sperm) glycolyze glucose, fructose, and mannose at about the same rate. The epididymal sperm differs from the ejaculated sperm in that it can utilize maltose only at an extremely slow rate.

The storage of excised epididymides in the refrigerator for one to two days results in an increased endogenous respiration rate of their contained spermatozoa. This phenomenon may be related to the natural "maturation" of spermatozoa in the epididymis.

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Studies of Fat and Carbohydrate Oxidation in Mammalian Spermatozoa *

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INTRODUCTION

In 1941 it was reported that the energy reserve of ejaculated bull spermatozoa is phospholipid in nature (1). Both the endogenous phospholipid and added phospholipids from a wide variety of sources were found to be utilized by an oxidative process for the maintenance of motility (2). Studies were immediately begun to determine the mechanism of lipid utilization. Although no conclusive pathway was established for the desmolysis of the carbon chain, it was recently established that the oxidation of the lipid reserve (1) resulted in the esterification of inorganic phosphate and the production of a calcium-insoluble, acid-labile phosphate ester which appeared to be adenosine-triphosphate (3). In regard to the pathway of oxidation, it was found that under certain conditions one could demonstrate citric acid production by enzyme preparations from bull spermatozoa and that, occasionally, the intact cells would accumulate citrate. In comparable experiments with minced rat kidney, treated with malonate, appreciably more citric acid would accumulate when lecithin was added than in control samples without added phospholipid. These results, together with the known facts of fat metabolism, indicated a possible pathway for fat oxidation. Breusch (4) has recently proposed that fat oxidation proceeds by the "citric acid cycle" but continues his opposition to the proposal that the cycle plays a part in the oxidation of carbohydrates. Experiments were therefore undertaken to determine

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which intermediary metabolites could be utilized by bull spermatozoa, and to determine whether the utilization of any of these would be prevented by malonate—an inhibitor of one of the essential steps of the "Krebs' Cycle." Comparative data were also obtained for the effect of 2,4-dinitrophenol on the utilization of the various metabolites in view of the observation that lipid utilization in spermatozoa is inhibited, while carbohydrate oxidation is stimulated, by this reagent (5).

METHODS AND MATERIALS

Sperm suspensions were prepared and respiration measured as described elsewhere (6, 3). Malonate and 2,4-dinitrophenol (DNP), when added, were present in a final concentration of 0.01 and 0.0001 *M* respectively. Motility of the spermatozoa from each flask was observed at the end of the experimental period. The suspension media (6) were buffered at pH 7.0 for ejaculated, and at pH 7.4 for epididymal spermatozoa in all experiments reported herein. At pH 7.0, 0.01 *M* malonate inhibited endogenous respiration of ejaculated spermatozoa about 55 per cent; the inhibition was greater in more acid, and less in more alkaline media.

The metabolites were as follows: Crystalline sodium pyruvate was prepared according to Robertson (7). Pure *d*- and *l*-lactic acids were kindly furnished by Dr. M. J. Johnson. Crystalline oxalacetic acid was prepared from the diethyl ester (8). Crystalline dimethyl isocitric acid lactone was supplied by Dr. V. R. Potter. The ester was saponified with the required amount of dilute sodium hydroxide on the steam bath and the methyl alcohol distilled off. *trans*-Aconitic acid was synthesized by Dr. H. Sober. From this, *cis*-aconitic acid anhydride was prepared by the procedure of Malachowski and Maslowski (9); it spontaneously yields *cis*-aconitic acid in aqueous solution. *dl*- β -Hydroxybutyrate was obtained from Dr. P. P. Cohen, acetoin from Mr. D. Blake, and α -hydroxyisobutyrate from Dr. A. L. Lehninger. Synthetic *l*- α -glycerophosphate was kindly furnished by Prof. H. O. L. Fischer and Dr. E. Baer. Egg lecithin was prepared as previously described (2). The other materials were the purest available commercially.

Lecithin, when added to the sperm suspensions, was used in a final concentration of 5 to 10 mg./cc.; all other metabolites were added to a final concentration of 0.01 *M*. All acids were neutralized to pH 7.0 with sodium hydroxide; oxalacetic acid just before each experiment.

Citric acid was determined by a procedure described elsewhere (10).

RESULTS

Metabolite Utilization

The effects of 21 different metabolites on the respiration and motility of normal, malonate-treated, and DNP-treated ejaculated bull spermatozoa in comparison with the endogenous respiration and motility under each of these respective treatments are shown in Table I.

The results are expressed as the average percentage of the endogenous respiration for the respective treatment in each individual experiment.

Carbohydrate Utilization. As observed previously (1), glucose depressed the endogenous respiration slightly, probably because its breakdown to lactic acid supplied energy and lessens the demand for oxidative energy. Pyruvate increased oxygen consumption of ejaculated spermatozoa above the endogenous rate, while lactate did not. Lactate was beneficial in maintaining the original rate of respiration, and both lactate and pyruvate prolonged motility. In epididymal spermatozoa each of these metabolites increased respiration and motility.

These carbohydrate metabolites lessened malonate inhibition. In the presence of DNP they greatly increased respiration and lessened the harmful effect of DNP on motility. Lactate and glucose increased respiration more than did pyruvate, indicating that the preliminary dehydrogenations, as well as the subsequent decarboxylative dehydrogenations, are accelerated by DNP.

Lactic acid of the *d* configuration is not utilized by spermatozoa under any of these conditions but does not prevent the utilization of the *l* component in a *dl* mixture.

Four-carbon Dicarboxylic Acids. The response of control specimens of ejaculated spermatozoa to additions of the four-carbon dicarboxylic acids varied considerably, but only oxalacetate produced appreciable increases in respiration and a consistent improvement in motility. Each of these was effective in reversing malonate inhibition of respiration and especially of motility, but only oxalacetate stimulated respiration and supported motility of DNP-treated spermatozoa. It is possible that oxalacetate was spontaneously or enzymatically decomposed to pyruvate and CO₂ and that, in reality, pyruvate was supporting respiration and motility in the DNP-treated specimens.

Tricarboxylic Acids. None of the tricarboxylic acids tried appreciably increased endogenous respiration or motility of ejaculated bull spermatozoa. Depression of the endogenous rate of respiration by citrate was slight, but consistent; motility, however, was usually slightly improved. The naturally-occurring tricarboxylic acids were utilized by epididymal spermatozoa and, in the ejaculated sperm they reversed malonate inhibition of respiration and motility. They were ineffective in preventing the detrimental effects of DNP.

Lipids and Their Intermediates. Phospholipids, though they increase respiration and prolong motility, do not prevent malonate or DNP

inhibition of motility or respiration. This is in agreement with the previous conclusion (2) that the natural lipid reserve of the sperm and added phospholipids are utilized in the same manner.

Acetate and acetoacetate increased the oxygen consumption of epididymal spermatozoa and these, as well as β -hydroxybutyrate, supported motility in both epididymal and ejaculated spermatozoa. In view of the fact that β -hydroxybutyrate depressed respiration of both epididymal and ejaculated spermatozoa but greatly improved motility, it seems likely that the oxidation of this metabolite must be more efficiently coupled with phosphorylation (or energy utilization) than the oxidation of the lipid reserve. These intermediates of fat metabolism differ sharply from the lipid reserve and from added egg phospholipids in that they greatly stimulate oxygen consumption and support motility in the presence of DNP. Of the various lipids and fat intermediates, only β -hydroxybutyrate consistently increased oxygen consumption and seemed to benefit motility in the presence of malonate; this will be discussed below.

The mechanism of action of DNP has been discussed elsewhere (5). Further insight, as to its mechanism of action, has been gained by the observation that this substance inhibits the oxidation of intact lipids, yet greatly increases the oxidation of acetate, acetoacetate, and β -hydroxybutyrate.

Acetoin, α -hydroxyisobutyrate and α -glycerophosphate, were not utilized, the latter probably, because it is a phosphorylated compound, does not enter the cell.

Citrate Production

It is well known that semen contains a high concentration of citrates (11), and in the present work it was found that both epididymal and ejaculated spermatozoa, even after several washings, contain appreciable quantities of citrate. Experiments with intact ejaculated spermatozoa showed that there was no accumulation of citrate during incubation either with or without additions of substrates or malonate. Epididymal spermatozoa, however, were found to produce appreciable quantities of citrate if pyruvate was present. Additions of malonate decreased the accumulation of citrate, and when malonate was added in the absence of pyruvate, the original content of citrate largely disappeared.

An aqueous extract of dried sperm proteins (prepared by treatment

of a washed sperm suspension with 2 volumes of acetone, centrifuging, and drying the residue in a vacuum) was found not to cause citrate to disappear above the amount which, it could be predicted (12), would form isocitric and *cis*-aconitic acids if aconitase were present. This extract was found to produce citrate if oxalacetate was added, but the production was not increased by the further addition of pyruvate.

The aqueous extract of sperm acetone powder contained aconitase as demonstrated by its ability to produce citric acid from either isocitric or *cis*-aconitic acids. The aconitase of the sperm differs from that in other tissues investigated (13) in that it is not destroyed by treatment with acetone. Extracts of rat muscle powders which had been prepared as described above were found to be devoid of aconitase activity.

This demonstrated ability of the sperm to produce citric acid must not be taken as evidence that the citric acid of seminal fluid is produced by the sperm. We have found that human ejaculates that were azoospermic¹ contained 8 to 9 mg. % of citric acid, a concentration which is in the higher brackets of the normal range for semen.

DISCUSSION

In spermatozoa the utilization of the endogenous lipid reserve, added phospholipids, acetate or acetoacetate is inhibited by malonate. The inhibition of respiration is reflected in an even greater degree of inhibition of motility. At low concentrations, malonate is considered to be fairly specific in its inhibition of the enzyme succinic dehydrogenase. This enzyme plays an essential part in both the Szent-Györgyi scheme of hydrogen transport by four-carbon dicarboxylic acids and in the Krebs isocitric acid cycle. While it can be demonstrated that certain four-carbon dicarboxylic acids can accept hydrogen from other metabolites under certain conditions, this pathway of hydrogen transport has been found not to be essential for the oxidation of triose phosphate by animal tissues (14), or the oxidation of alcohol to acetic acid by yeast (15). Furthermore, Ochoa (16) found that the oxidation of α -ketoglutarate to succinate did not require the addition of a four-carbon acid, and there is an indication, in the results shown in Table I, that the oxidation of β -hydroxybutyrate to acetoacetate not only

¹ These specimens were from a patient who had had bilateral orchitis during mumps and were kindly made available by Dr. Elmer L. Sevringhaus.

TABLE I
Utilization of Various Substrates by Normal, Malonate-treated, and DNP-treated Ejaculated Bull Spermatozoa

Metabolite	No. of Expts.	Control		0.01 M Malonate		0.0001 M DNP	
		No. of Expts.	Motility at 1st hr.	No. of Expts.	Motility at 2 hrs.	No. of Expts.	Motility at 2 hrs.
Endogenous	17	17.0	3+	10	7.7	7.3	V.F.M.
		% of endogenous rate			% of malonate-treated rate	% of DNP-treated rate	
Glucose	6	89	4+	3	148	448	3-4+
Pyruvate	5	118	4-5+	4	156	228	2-3+
<i>L</i> -Lactate	3	109	4-5+			607	2-3+
<i>D</i> -Lactate	2	100	3+			100	V.F.M.
<i>DL</i> -Lactate	2	110	4-5+			570	2-3+
Succinate	2	98	3-4+	3	170	125	V.F.M.
Fumarate	2	126	3-4+	3	155	75	V.F.M.
Malate	4	122	3-4+	4	149	91	V.F.M.
Oxalacetate	2	227	4+	2	171	210	2+
Citrate	5	90	3-4+	4	196	125	V.F.M.
Isocitrate	2	102	3+	2	170	90	V.F.M.
<i>cis</i> -Aconitate	3	93	3+	6	182	114	V.F.M.
<i>trans</i> -Aconitate	3	70	3+	3	107	110	V.F.M.
Acetate	3	98	5+	2	84	246	2+
Propionate	2	94	3+	1	128*	150*	V.F.M.
Butyrate	1	135	1+	1	135*	180	F.M.
β -Hydroxybutyrate	3	71	5+	3	128	268	2+
Acetoacetate	2	83	5+	1	90	248	2+
Acetoin	1	108	3+			109	V.F.M.
Egg phospholipid	2	140	5+	2	104	106	V.F.M.
α -Glycerophosphate	2	105	3+			125*	V.F.M.
α -Hydroxyisobutyrate	1	100	3+			91	0

* Because of the low endogenous respiration of the accompanying control these increases are not significant.

occurs in the presence of malonate, but also supports motility. The majority of experimental evidence is in agreement with the view that malonate inhibits tissue oxidations by inhibiting an essential step in the isocitric acid cycle (see 17). Our results are in agreement with the theory that both fats and carbohydrates are metabolized through such a cycle. Breusch (4) has proposed that β -keto acids, but not carbohydrates, are metabolized through the citric acid cycle. Pyruvate (and glucose) increases respiration and improves motility of malonate-treated spermatozoa. However, pyruvate is also utilized in malonate-treated pigeon liver preparations (18). This utilization of pyruvate by malonate-treated spermatozoa may be possible because of the condensation of pyruvate and CO_2 within the cell as in the case of pigeon liver (19).

The demonstrated ability of spermatozoa, and enzyme preparations therefrom, under certain conditions, to utilize citrate, isocitrate, *cis*-aconitate and the four-carbon dicarboxylic acids of the Krebs cycle, and to form citric acid from such metabolites as pyruvate and oxalacetate, together with the finding that fat oxidation by spermatozoa is inhibited by malonate, lends support to the hypothesis that fat, as well as carbohydrate, is oxidized stepwise through the isocitric acid cycle.

The inability of the tricarboxylic acids and succinate, fumarate and malate to increase respiration or prolong motility of *untreated* ejaculated bull spermatozoa may be the result of the spermatozoa being relatively impermeable to them. Some bacteria (20, 21), yeast (15), and the erythrocyte (22) are relatively impermeable to salts of some of these acids, and one can reason that it is advantageous to the cells to be so. These acids are probably intermediates in the scheme of carbohydrate, amino acid, and fat metabolism and, were these unicellular forms permeable to them, they would either diffuse out, requiring more to be formed, or an energy requiring mechanism would be necessary to retain them within the cell.

SUMMARY

A study of the utilization of various intermediary metabolites by bull spermatozoa has been made using respiration and maintenance of motility as criteria of the ability of these metabolites to furnish energy under various experimental conditions.

Glucose, pyruvate, *l*-lactate were utilized by epididymal, and by malonate-treated, and 2,4-dinitrophenol-treated ejaculated spermatozoa.

Succinate, fumarate, malate were not utilized by ejaculated bull spermatozoa. These 4-carbon acids and oxalacetate were effective in reversing malonate inhibition of motility and respiration. Oxalacetate improved motility and respiration of normal bull spermatozoa and was the only 4-carbon dicarboxylic acid effective in increasing respiration and supporting motility of dinitrophenol-treated spermatozoa. Its efficacy may have been the result of decarboxylation to pyruvate which is utilized in both cases.

Egg phospholipids increased respiration and prolonged motility but were without effect on malonate or dinitrophenol inhibition of endogenous respiration and motility. Acetate, acetoacetate, and β -hydroxybutyrate did not stimulate respiration but prolonged maintenance of motility. They support both respiration and motility of dinitrophenol-treated spermatozoa. β -Hydroxybutyrate alone, among these fat intermediates, consistently increased respiration and improved motility of malonate-treated spermatozoa. It seems probable that the oxidation of β -hydroxybutyrate to acetoacetate supported motility in this case.

Acetoin, propionate, butyrate, α -glycerophosphate, α -hydroxyisobutyrate, *d*-lactate were not utilized for the maintenance of motility.

Spermatozoa and enzyme preparations therefrom can synthesize citric acid from pyruvate or oxalacetate under certain conditions. The enzyme aconitase is present in acetone-dried spermatozoa and may be extracted into aqueous solution.

The data lend support to the hypothesis that fats, as well as carbohydrates, are metabolized through the isocitric acid cycle.

We wish to express our sincere appreciation to the National Committee on Maternal Health Inc. for the support of these investigations and to Dr. Elmer L. Sevringhaus for his kind cooperation.

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Studies on the Metabolism of Nicotinic Acid in the Horse *

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INTRODUCTION

While the horse was among the earliest animals domesticated by man, relatively little is known about the quantitative requirements of this species for the various vitamins of the B group. It has been shown that the horse differs from ruminants in that it requires certain members of the B vitamins (1, 2).

During the course of experiments designed to determine whether or not various members of the B vitamins are dietary essentials for the horse it was observed that the amounts of riboflavin and pantothenic acid that were excreted in the urine might vary by 50 fold when the amount in the diet was changed from an adequate to a low level as judged from known requirements of other species. The amount of nicotinic acid excreted in the urine was, however, influenced only slightly by different dietary regimens to which the animals were subjected. In species for which nicotinic acid is a dietary essential, such as the dog and man, the amount of nicotinic acid and derivatives excreted in the urine varies with the amount ingested (3-6). These facts suggested to us that nicotinic acid may be synthesized in the body of the horse. Another possibility of accounting for the minor changes in the urinary excretion of nicotinic acid when the animals were changed from a low to an adequate level is that the ingested nicotinic acid may be excreted as derivatives such as trigonelline or *N*-methyl nicotinamide which do not stimulate the growth of *Lactobacillus arabinosus*.

* The authors are indebted to Merck & Company for a generous supply of nicotinic acid used in these investigations.

We were primarily interested in determining whether or not nicotinic acid is synthesized in the body of the horse, and the effect of the ingestion of large quantities of nicotinic acid on the excretion of nicotinic acid and certain methylated derivatives in the urine.

EXPERIMENTAL

Three groups of Shetland ponies have been used during the course of these studies. They were fed slight variations of a basal diet made up of dried beet pulp, casein, corn, minerals, supplements of vitamins A and D, and brewers' yeast or synthetic B vitamins.

Nicotinic acid or brewers' yeast was added to the rations in amounts to furnish approximately 0.25 and 0.5 mg. of nicotinic acid per kg. of body weight per day. The source of the nicotinic acid had no bearing on the performance of the animals provided adequate amounts of the other B vitamins were furnished. The basal ration which contained no added source of nicotinic acid provided approximately 0.10 mg. of nicotinic acid per kg. of body weight per day. This is considerably less than the minimum daily requirement of 0.25 mg. per kg. of body weight for the dog (7), pig (8) and human (9).

Weanling Shetland ponies were fed rations which provided approximately 0.10, 0.25, and 0.50 mg. of nicotinic acid per kg. of body weight per day. The rate of growth of the ponies receiving the three different levels of nicotinic acid was virtually the same.

Balance Studies. Since the growth of ponies was not affected by a low intake of nicotinic acid, an experiment was designed to determine whether or not nicotinic acid is synthesized in the body of the horse. Four Shetland ponies approximately six months of age were fed a ration of the following percentage composition: dried beet pulp 58, yellow corn 31, casein 10, dicalcium phosphate 1, sodium chloride *ad libitum*, A and D vitamin concentrate, and synthetic riboflavin and calcium pantothenate at levels estimated to be adequate to satisfy the dietary requirements.

The ponies fed this ration for a period of 35 weeks made satisfactory growth. At the end of the period the feces and urine were collected quantitatively over a two-day period. The corn in the ration was then replaced by glucose. This reduced the daily nicotinic acid intake to approximately 0.01 mg. per kg. of body weight. After five weeks on this ration the feces and urine were again collected. The feces, urine and feed were assayed for nicotinic acid by the method of Krehl, Strong, and Elvehjem (10).

The intake and excretion of nicotinic acid of the horses while on the 0.10 and 0.01 mg. per kg. of body weight per day levels of nicotinic acid are shown in Table I. The daily nicotinic acid intake is based on the average amount of feed consumed for the two days prior to the collection period plus the average for the two days the ponies were in the metabolism cage. This was done to avoid any residual effect from differences in feed consumption.

TABLE I
Intake and Excretion of Nicotinic Acid

Experiment No. 1 Daily nicotinic acid intake 0.10 mg. per kg. body weight					Experiment No. 2 Daily nicotinic acid intake 0.01 mg. per kg. body weight			
Animal no.	Intake per day	Daily excretion			Intake per day	Daily excretion		
		Feces	Urine	Total		Feces	Urine	Total
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	9.51	4.25	1.13	5.38	1.07	1.17	1.54	2.71
2	11.10	3.49	1.78	5.27	1.07	1.53	1.87	3.39
3	11.10	9.60	2.10	11.70	1.07	2.99	2.32	5.31
4	11.10	3.21	2.17	5.38	1.07	9.83	2.85	12.68

The urinary excretion of nicotinic acid did not decrease when the intake was reduced from 0.10 to 0.01 mg. per kg. of body weight per day. Had the synthesis of nicotinic acid not occurred, the urinary excretion could be expected to show a significant decrease such as has been shown to occur with the dog and human (3-6). In the dog, guinea pig, and man, feeding an inadequate amount of nicotinic acid is followed by the virtual disappearance of nicotinic acid from the urine. Evidence that the length of the feeding period with the horses was sufficiently long is adduced from work with dogs (3). Nicotinic acid was not found in the urine of dogs after 28 days on a deficient diet. Within four weeks after horses are placed on a riboflavin-deficient diet the amount of riboflavin excreted in the urine declines to a rather constant level (1). The amount excreted may even be too small to measure microbiologically.

Reducing the daily intake of nicotinic acid from about 10 mg. to 1.07 mg. resulted, as might be expected, in a decrease in the fecal excretion of nicotinic acid. The decrease in the nicotinic acid content

of the feces was not, however, proportional to the decrease in the amount ingested.

An examination of the data under experiment 2, Table I shows that the daily urinary excretion of nicotinic acid in feces and in the urine each exceeded the intake. The extra nicotinic acid excreted over that ingested must have been synthesized. The only other source would have been from metabolized body tissues. Since ponies 1, 2, and 4 showed a slight gain in weight during experiment 2, the evidence is against the origin of the extra nicotinic acid from body tissues. The fact that more nicotinic acid was excreted in the feces than was ingested indicates that nicotinic acid is synthesized by the symbiotic action of microorganisms in the gastrointestinal tract of the horse.

Effect of Ingestion of Excessive Amounts of Nicotinic Acid. Five grams of nicotinic acid were added to the daily ration of each of four

TABLE II
Recovery of Ingested Nicotinic Acid

Animal no.	Nicotinic acid ingested g./24 hrs.	Urinary nicotinic acid Total g./24 hrs.	Recovered per cent
5	5.00	2.15	43.3
6	5.00	3.10	60.2
7	5.00	1.59	31.8
8	5.00	1.66	33.3
9	5.00	2.24	44.9

different ponies for four consecutive days. The urine was collected on the third and fourth days. The urinary excretion of nicotinic acid following the ingestion of five grams of nicotinic acid is reported in Table II. The amount of nicotinic acid contributed by the basal ration (0.10 mg. per kg. body weight daily) was so small that it would make no difference in the per cent recovered. For this reason the calculations in Table II are based only on the synthetic nicotinic acid ingested. The per cent of ingested nicotinic acid which was excreted in the urine ranged from 31.4 to 60.2. This is a considerably larger amount than can be accounted for with normal men following the ingestion of nicotinic acid or nicotinamide. Sarett, *et al.* (11) measured the total urinary nicotinic acid and its derivatives in human urine and accounted for only about 34% of the ingested synthetic vitamin.

A relatively large per cent of ingested nicotinic acid is excreted by the rat (12), dog (13), and human (11) as trigonelline or *N*-methyl

nicotinamide. Tests were made on the urine of horses to determine whether or not these are metabolites of nicotinic acid in the horse. The urine from the horses fed either the basal diet with or without the addition of the five grams of nicotinic acid did not give a positive test for trigonelline by the method of Kodicek and Wang (14) or *N*-methyl nicotinamide or trigonelline by the method of Sarett (15). These derivatives could not be detected in equine urine even when the amount of urine used was five times the amount needed for a quantitative measure in the urine of humans on a normal diet. Trigonelline and *N*-methyl nicotinamide are not end products of nicotinic acid metabolism in the rabbit (12). Thus it appears that the end products of nicotinic acid metabolism and excretion of the horse may be similar to those of the rabbit but different from those of the rat, dog, and man.

SUMMARY

Horses made normal growth on a diet that provided approximately 0.10 mg. of nicotinic acid per kg. of body weight per day. When the daily intake of nicotinic acid was reduced to 0.01 mg. per kg. of body weight the amount excreted in the urine and the feces each exceeded the intake. On the basis of these data it appears that nicotinic acid is synthesized in the body of the horse and that this vitamin is not a dietary essential for the horse.

When five grams of nicotinic acid were fed daily an average of 43% was excreted in the urine. Neither trigonelline nor *N*-methyl nicotinamide are the principal end products of metabolism in the horse as they are in the dog, man, and rat.

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Prothrombase and Thrombase *

I. Preparation and Purification

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INTRODUCTION

Numerous methods for the preparation and purification of prothrombase and thrombase have been described (1-16). This paper describes a modification of the Mellanby method (5) in which prothrombase is precipitated from diluted plasma by acetic acid at a pH of 5.3. The modified method gives rapid and consistent results.

EXPERIMENTAL

Analytical Methods. Thrombase Assay. 0.1 cc. of the thrombase solution is rapidly added to 1 cc. of fresh oxalated beef plasma in a 10 × 75 mm. tube at 37°C. and thoroughly mixed by inversion of the tube. The clotting time is the point at which the first sign of opalescence appears in the tube when viewed in a bright light. One thrombase unit is the concentration which will produce a clot in 30 seconds. The solutions are diluted out until this clotting time is obtained. The activity is expressed in units per mg. of N.

Determination of Protein Nitrogen in Solutions Containing $(\text{NH}_4)_2\text{SO}_4$. This method is a modification of that described by Sørensen and Sørensen (17), using the micro-Kjeldahl technique. The entire operation is performed in a 100 cc. Pyrex centrifuge tube which is graduated at 35 cc. and 50 cc.

To X cc. of the protein solution, which contains $(\text{NH}_4)_2\text{SO}_4$, is added (50-X) cc. distilled water and 25 cc. saturated KNaSO_4 solution, after which is added 5 cc. of the tannic acid reagent¹ with good mixing. After standing until the next day, the precipitate is centrifuged off (15 minutes at 3000 r.p.m.) and washed carefully by

* Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

¹ *Tannic Acid Reagent.* 70 g. tannic acid and 100 g. NaCl are dissolved in water, 50 cc. glacial acetic acid added and the whole made up to one liter with water.

centrifugation with several 35 cc. portions of a solution containing 10 cc. tannic acid reagent and 50 cc. saturated KNaSO_4 per liter. The final precipitate is centrifuged off and the nitrogen determined by the micro-Kjeldahl technique. All operations are carried out at room temperature.

Preparation of Prothrombase and Thrombase. Beef blood is drawn directly into vessels containing 10 cc. of 20% potassium oxalate for each liter of blood. The plasma is obtained by centrifugation at 3500 r.p.m. To secure a sufficiently large yield of thrombase at least 1600 cc. of plasma should be used and the preparation started within 24–48 hours after blood is drawn. The yield of thrombase obtained decreases with the age of the plasma. The plasma is diluted with 9 volumes of distilled water and the resultant solution is brought to a pH of 5.2–5.4, as measured with the glass electrode, with 1% acetic acid. A flocculent precipitate forms which settles in from one to one-and-one-half hours. The supernatant liquid is syphoned off and the precipitate centrifuged at 2500 r.p.m. for 10 minutes. In this way, the precipitate is obtained as a compact mass, free from most of the albumin and consists of the prothrombase associated with fibrinogen, globulins, and lipids.

This precipitate is triturated with small quantities of distilled water until a smooth suspension having a volume of about 800 cc. is obtained. The suspension is thoroughly mixed with an equal volume of a buffer which was prepared as follows: 35 cc. of $M/15 \text{ Na}_2\text{HPO}_4$ and 15 cc. of $M/15 \text{ KH}_2\text{PO}_4$ are diluted to 990 cc. with distilled water and 10 cc. of 1% CaCl_2 solution is added to the mixture. It has been found by a series of experiments that a buffer of this salt concentration at this pH extracts the maximum amount of prothrombase with a minimum quantity of inactive proteins.

For the best results, the pH of the mixture of the suspension plus the buffer should fall within 6.2–6.5. If necessary the pH is adjusted to this range with 1% acetic acid or 0.5% Na_2CO_3 . The suspension is stirred frequently for 10 minutes, centrifuged at 2500 r.p.m. for 15 minutes and the supernatant liquid filtered. The prothrombase is precipitated from this filtrate at pH 5.0–5.1 by addition of 1% acetic acid. The suspension is centrifuged at 2500 r.p.m. for 10 minutes and the supernatant liquid poured off, leaving about 1–2 cc. remaining in contact with the precipitate I. The wet prothrombase precipitate, standing at room temperature for approximately 24 hours, is converted into thrombase (to prepare prothrombase, precipitate I is immediately dried with acetone). This suspension of thrombase is added to several volumes of cold, redistilled acetone, allowed to stand 15 minutes at 4°C. and filtered with suction on a Buchner funnel through Whatman No. 50 filter paper. The precipitated thrombase is washed with cold acetone, pulverized and dried in vacuo. The average yield from 1600 cc. plasma varies between 0.8–1.2 g.

The results of assays on a number of crude thrombase preparations

TABLE I

Assay of Crude Thrombase Preparations

Experiment no.	Units per cc.	mg. N per cc.	Units per mg. N	Total units per preparation
1	79	.35	226	7,200
2	131	.59	222	14,400
3	290	.72	402	20,000
4	88	.57	154	6,400
5	88	.33	266	8,000
6	79	.78	101	11,000

TABLE II

The Activity of Solutions of the Precipitates Obtained from Thrombase A Solutions by $(\text{NH}_4)_2\text{SO}_4$ at 0-46%, 46-64% and 64-100% Saturation

Experiment no.	% Saturation	Units per cc.	mg. N* per cc.	Units per mg. N
1	0-46	5	.18	28
	46-64	50	.07	714
	64-100	0	.07	0
2	0-46	18	.33	55
	46-64	170	.18	944
	64-100	0	.13	0
3	0-46	5	.20	25
	46-64	100	.10	1000
	64-100	0	.07	0

* Protein nitrogen was determined by modification of method of Sørensen and Sørensen (17).

are summarized in Table I. The solutions to be assayed are prepared by suspending 50 mg. of crude thrombase in 5 cc. distilled water, the pH is adjusted to 7.0 with 0.5% Na_2CO_3 and the mixture is centrifuged. The supernatant liquid is used in the assay. The activity ranges from 101-402 units per mg. of N and 6400-20,000 units per preparation. One unit of activity is the amount of thrombase required to clot 1 cc. of fresh oxalated beef plasma in 30 seconds at 37°C.

This modification of the Mellanby method is rapid, giving consistent results with oxalated and citrated beef and oxalated sheep plasmas. Thus far it has not been possible to prepare thrombase by this method from oxalated or citrated human or oxalated hog plasmas. Preliminary experiments with human and hog plasmas indicate that the precipitation limits or isoelectric points for prothrombase in these species are different from the precipitation limits found in beef and sheep plasmas.

Active material has been prepared without calcium ions in the buffer solution tending to show that calcium may not be necessary but those preparations containing calcium ions were more active, indicating that calcium accelerates the conversion.

The conversion of prothrombase to thrombase by this method is not quantitative, further activation occurs upon addition of thromboplastin (acetone dehydrated rabbit brain extracted with 0.9% NaCl) plus calcium ions. The differences in activity of the preparations shown in Table I may be due to differences in the amount of thromboplastin which is carried along in the preparation.

Purification of Thrombase. One gram of the crude thrombase is suspended in 50 cc. distilled water, mixed thoroughly and the pH adjusted to 5.0–5.1 with 1% acetic acid. The mixture is allowed to stand 15 minutes with frequent stirring, centrifuged and the undissolved residue re-extracted with small portions of distilled water at pH 5.0 until all the thrombase activity is removed. These extracts are combined and filtered (thrombase A). The residue still contains active material which is not extracted by water. There may be two different thrombases with different solubilities (Seegers, 13). Thrombase A solutions can be stored at 4°C. for months without appreciable loss of activity when preserved with merthiolate 1:10,000. Solutions can also be lyophilized and stored in the dried state.

Thrombase A solutions were separated into precipitates obtained by $(\text{NH}_4)_2\text{SO}_4$ at 0–46%, 46–64% and 64–100% saturation. The precipitates were filtered using Whatman No. 50 filter paper, dissolved in water and assayed. The results of several experiments are summarized in Table II. The activity was concentrated into the 46–64% fraction (thrombase B); the highest activity obtained was 1000 units per mg. of N.

Dialysis of the thrombase B solutions to remove the $(\text{NH}_4)_2\text{SO}_4$ for several days against distilled water at 4°C., using either cellophane or Visking casings, showed a great diminution in the activity. No activity could be detected in the dialyzate. The undialyzed fraction can be either lyophilized or stored at 4°C. for months without any appreciable loss in activity when preserved with merthiolate 1:10,000.

DISCUSSION OF RESULTS

The precipitation of prothrombase and associated plasma proteins by a weak acid gives the most active preparations of thrombase. The

primary differences in the various methods consist in the use of different agents to extract the prothrombase from the precipitate. It has been shown that the special buffer solution described is a very effective agent for the extraction and easily prepared. The (neutral) $\text{Ca}(\text{OH})_2$ solution which is essential for the success of the Mellanby method is difficult to prepare with the result that active preparations of thrombase are not always obtained. With this modified method, very active preparations of thrombase can be prepared consistently in 24–36 hours. The method yields thrombase from blood plasma without the addition of any biological extracts.

Astrup and Darling (7) on the basis of precipitation experiments with $(\text{NH}_4)_2\text{SO}_4$ classified thrombase as an albumin. Their most potent fraction contained 1330 units per mg. of N. Their thrombase unit is defined as the amount of active material which will clot 1 cc. oxalated plasma in 30 seconds at 37°C . Seegers and McGinty (13) have recently reported studies on highly purified preparations. Their most potent products prepared averaged 11,492 units per mg. of N. Solubility studies, using Na_2SO_4 , show two active components with different solubilities: 10,086 and 13,365 units per mg. of N. Their thrombase unit is defined as the amount of active material required to clot 1 cc. of a standard fibrinogen solution in 15 seconds at 28°C . In their recent work using oxalated plasma, they found 2.25–2.5 units of thrombase will clot 1 cc. of oxalated beef plasma in 15 seconds at 28°C . Milstone (12), using the Mellanby method, combined with $(\text{NH}_4)_2\text{SO}_4$ fractionation, obtained activities as high as 36,000 units per mg. of N. One unit is the amount of thrombase which will give a clotting time of 300 seconds using a purified fibrinogen solution at room temperature. This unit is equal to one-sixth of the Seegers unit. He found thrombase to be soluble in 0.45 saturated $(\text{NH}_4)_2\text{SO}_4$. Solubility and electrophoretic data indicated inhomogeneity.

The difficulty in following the work on thrombase lies in the variety of assay methods. In the investigations discussed, three different thrombase units have been described. The assay methods should be standardized so that experimental results of different investigators may be compared.

SUMMARY

1. A rapid, reliable, modified Mellanby method for the preparation of prothrombase and thrombase is described.

2. This method is applicable to oxalated and citrated beef and oxalated sheep plasmas but is not applicable to oxalated or citrated human or oxalated hog plasma.

3. When thrombase is fractionated with $(\text{NH}_4)_2\text{SO}_4$, the activity is found in the fraction precipitated by 46-64% saturation with ammonium sulfate; the most active preparation contains 1000 units per mg. of N.

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Prothrombase and Thrombase *

II. Chemical Composition, Relationship, and Chemistry of the Activation of Prothrombase

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INTRODUCTION

The relationship between prothrombase and thrombase has not been investigated sufficiently from a chemical or physical viewpoint to have much significance (1-11). The differentiation between the two compounds is based solely on activity or solubility of their protein combinations.

The purpose of this investigation is to study the chemical composition of prothrombase and thrombase and the relationship between these compounds utilizing immunological and chemical methods. On the basis of these results, the chemistry of the activation of prothrombase will be discussed.

METHODS

Preparation of Antigens. Beef prothrombase and thrombase (B) were prepared according to the method described by Robbins (7). The prothrombase was purified by extraction of the acetone dried precipitate with distilled water at pH 7.0. The prothrombase (A) is precipitated from this solution at pH 5.0-5.1 with 1% acetic acid and the precipitate redissolved in distilled water at pH 7.0. Freshly prepared solutions may gel upon standing but upon removal of the coagulum by centrifugation, the prothrombase solutions are stable for months when preserved with merthiolate 1:10,000 at 4°C. The prothrombase (A) solutions were made up to contain 1.0 mg. of protein N per cc. The thrombase (B) solutions were those prepared by fractionation at 46-64% saturation with $(\text{NH}_4)_2\text{SO}_4$ and preserved with merthiolate 1:10,000 at 4°C.

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Beef albumin, pseudoglobulin, and euglobulin were prepared according to the methods described by Hektoen and Welker (12) with modifications (13). Solutions of albumin and pseudoglobulin were prepared in 0.9% NaCl, the euglobulin in 10% NaCl. These solutions were made up to contain 1.0 mg. of protein N per cc. The pH of the solutions were adjusted to 6.5-7.0 and preserved with merthiolate 1:10,000 at 4°C. The beef fibrinogen was prepared according to the method described by McLean (14) with modifications (13). The fibrinogen was precipitated from oxalated plasma at 20% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in a 2% NaCl solution containing 0.2% $\text{K}_2\text{C}_2\text{O}_4$, and the fibrinogen was precipitated at 18% saturation with $(\text{NH}_4)_2\text{SO}_4$ from that solution. The latter process was repeated 6-8 times. The final precipitate was dissolved in the 2% NaCl solution containing 0.2% $\text{K}_2\text{C}_2\text{O}_4$ and this solution was made up to contain 1.0 mg. of protein N per cc. This method yields fibrinogen solutions which are stable for months at a pH of 5.7-5.9 when preserved with toluol at 4°C. aside of a slight precipitate which appears on standing. The albumin, pseudoglobulin, euglobulin, and fibrinogen solutions used as antigens were free from thrombase and prothrombase activities.

TABLE I

Relationship between Precipitin Titer and Concentration of Protein Nitrogen in Antigen Solutions

Precipitin titer	Concentration of protein nitrogen in antigen solutions
0	No reaction
1	1.0 mg. protein N per cc.
2	0.1 " " " " "
3	0.01 " " " " "
4	0.001 " " " " "
5	0.0001 " " " " "

Preparation of Antisera. Precipitin antisera were prepared in rabbits against prothrombase, thrombase, pseudoglobulin, and fibrinogen by intramuscular injections of the antigens adsorbed on aluminum hydroxide cream according to the method described by Hektoen and Welker (15). This method consists in the addition of one and one-half volumes of aluminum hydroxide cream (contains approximately 0.4 g. % Al_2O_3) (16) to one volume of a 1% protein solution. The protein adsorbed on the aluminum hydroxide is centrifuged off and the precipitate mixed with a sufficient volume of 0.9% NaCl solution to give a resultant suspension that can be handled in an 18 gauge hypodermic needle. 20-30 cc. of this suspension are injected into the thigh muscles of rabbits in depots of not more than 1-2 cc. Larger volumes in the depots tend to produce pressure necrosis. The prothrombase and thrombase are both quantitatively adsorbed by the aluminum hydroxide cream preparation (pH 4.2-4.3) (16).

The precipitin tests were made by the contact method with undiluted serum and progressive dilutions of the antigens in 0.9% NaCl solution (pH 7.0) and the results were read after one hour at room temperature.

The approximate titers of the precipitin sera were determined by finding the highest dilutions of the antigen with which definite precipitates would form under

the conditions stated. Titers are expressed in weights of protein nitrogen in the antigen solutions used, as illustrated in Table I. Fibrinogen solutions show false precipitins, due to clotting at the interface, in dilutions 1 and 2. Prothrombase does not give a reaction in dilution 1 because the original solution is in water. Thrombase solutions do not give reactions in dilutions 1 and 2 because the most concentrated solutions used contain only approximately 0.1 mg. of protein N per cc. The first dilution has a concentration of approximately 0.01 mg. of protein N per cc. Therefore either 3 or 0 will indicate the titer for thrombase. In certain instances, the more concentrated prothrombase solutions can be used to replace the thrombase solution. The original solution of euglobulin is in 10% NaCl and therefore does not give a true precipitin reaction when undiluted.

RESULTS

Prothrombase. Four rabbits each were given injections of beef prothrombase (A); 20–35 mg. of protein N was injected in each animal. The results of a typical experiment are illustrated in Table II. Pro-

TABLE II

Production of Precipitins by a Rabbit After a Single Intramuscular Injection of Beef Prothrombase (A) Adsorbed on Aluminum Hydroxide Cream*

Weeks after injection	Precipitin titers					Prothrom- base (A)	Throm- base (B)
	Albu- min	Pseudo- globulin	Euglob- ulin	Fibrin- ogen			
2	0	4	3	4		4	3
3	4	4	3	3		4	3
5	0	0	3	4		4	3
6	0	0	3	4		4	3
9	0	0	3	4		4	3
10	0	0	3	4		4	3
12	0	0	3	4		4	0
13	0	0	3	4		4	3
16	0	0	3	4		4	3
20	0	0	3	3		4	3
24	0	0	0	0		0	0

* 20 mg. of protein N injected.

thrombase (A) produces precipitins which react with prothrombase, thrombase, euglobulin, and fibrinogen in high titers. Two of the animals injected produced weak precipitins to pseudoglobulin which persisted only for three weeks. Pseudoglobulin may either exist as an impurity in the prothrombase preparations, or the pseudoglobulin preparation used as antigen may contain small amounts of euglobulin. Weak,

transitory precipitins to albumin appeared in two of the experiments. The precipitins to prothrombase, thrombase, euglobulin, and fibrinogen persisted from 4-5 months, depending upon the amount of protein injected. The disappearance of the precipitins to prothrombase and thrombase coincided with the disappearance of the precipitins to euglobulin and fibrinogen. These results indicate that prothrombase is composed of fibrinogen, euglobulin, and perhaps some other protein.

Thrombase. Seven rabbits each were given injections of beef thrombase (B); 1.4-5.4 mg. of protein N (900-5100 units) was injected in each animal. The results of a typical experiment are illustrated in Table III. Thrombase (B) produces precipitins which react with

TABLE III

Production of Precipitins by a Rabbit After a Single Intramuscular Injection of Beef Thrombase (B) Adsorbed on Aluminum Hydroxide Cream*

Weeks after injection	Precipitin titers				Prothrom- base (A)	Throm- base (B)
	Albu- min	Pseudo- globulin	Euglob- ulin	Fibrin- ogen		
2	0	2	3	3	4	3
3	0	0	3	3	4	3
4	0	0	3	3	4	3
5	0	0	3	0	4	3
6	0	0	3	0	4	3
8	0	0	3	0	4	3
10	0	0	3	0	3	3
12	0	0	0	0	0	0
14	0	0	0	0	0	0

* 3.0 mg. of protein N injected (3000 units).

thrombase, prothrombase, and euglobulin in high titers. Weak, transitory precipitins to fibrinogen appeared in three of the experiments, either alone or accompanied by weak, transitory precipitins to pseudoglobulin and albumin. Since prothrombase and thrombase both contain a common protein, namely, euglobulin, prothrombase was used as the antigen in those experiments where low titer precipitin sera were obtained. This is necessary because the protein content of the thrombase (B) solutions is low, titers below 3 cannot be detected. The appearance of weak, transitory precipitins to fibrinogen, pseudoglobulin, and albumin indicates that these proteins are probably present as impurities in the thrombase preparation. The precipitins to thrombase, prothrombase, and euglobulin persisted from 2-3 months,

depending upon the amount of protein injected. The disappearance of the precipitin to prothrombase and thrombase coincided with the disappearance of the precipitin to euglobulin. These results indicate that either thrombase is euglobulin or composed of euglobulin plus some other protein.

In Vitro Adsorption. In an attempt to prove whether or not thrombase is a specific protein and not euglobulin, *in vitro* adsorption experiments were carried out using euglobulin solutions and several thrombase precipitin sera. In these experiments, varying amounts of euglobulin were added to 1 cc. of the antiserum, and the mixtures were allowed to stand at room temperature for 24 hours. The precipitate which formed was centrifuged off and the supernatant liquid used for the precipitin tests. The euglobulin removes all the precipitins; the results of a typical experiment are shown in Table IV. These

TABLE IV
In Vitro Adsorption of Thrombase Precipitin Sera with Euglobulin

Antigens	Before adsorption	Precipitin titers Amount of euglobulin added in mg. of protein N			
		.0002	.002	.02	.04
Albumin	0	0	0	0	0
Pseudoglobulin	0	0	0	0	0
Euglobulin	2	2	2	0	0
Fibrinogen	0	0	0	0	0
Prothrombase (A)	3	3	3	0	0
Thrombase (B)	3	3	3	0	0

experiments indicate that euglobulin and thrombase are either identical or exist in combination.

Specificity. In order to determine the species specificity of thrombase, several thrombase precipitin sera were titrated against beef plasma and serum, human, dog, hog, and sheep sera. The sera and plasma were diluted 1:10 in order that proper stratification could be attained in the precipitin reaction. The results are summarized in Table V. The thrombase antisera reacted only with the homologous blood proteins (beef plasma, serum, and euglobulin). Beef hemoglobin was also included as a control.

This experiment indicates that the antigen or antigens in thrombase are species specific. This may mean that euglobulin is the carrier for the enzyme and therefore would be specific for each species of blood used although the coagulation activity is not specific.

TABLE V

*Precipitin Reactions of Homologous and Heterologous Blood Proteins with
Thrombase Precipitin Sera*

Antigens	Normal rabbit serum	Precipitin titers Serums of rabbits given injections of	
		Thrombase (B) 3*	Thrombase (B) 4*
Beef Albumin	0	0	0
" Pseudoglobulin	0	0	0
" Euglobulin	0	3	3
" Fibrinogen	0	0	0
" Serum	0	5	5
" Plasma	0	5	5
" Hemoglobin	0	0	0
" Prothrombase (A)	0	3	3
" Thrombase (B)	0	3	3
Human Serum	0	0	0
Sheep Serum	0	0	0
Dog Serum	0	0	0
Hog Serum	0	0	0

* Preparation number.

The thrombase precipitin reacted with beef serum, plasma, and euglobulin in high titer. This proves that the euglobulin part of the thrombase complex has not been denatured or modified by the methods of extraction and purification and, by virtue of the coagulation activity, that the thrombase part of the complex is not denatured.

*Fractionation of Prothrombase with a Neutral Saturated
Ammonium Sulfate Solution*

A freshly prepared prothrombase (A) solution was fractionated with a neutral saturated $(\text{NH}_4)_2\text{SO}_4$ solution (16 g. anhydrous Na_2HPO_4 per liter of saturated $(\text{NH}_4)_2\text{SO}_4$ solution, pH 6.9-7.0). Precipitates were obtained at 0-25%, 25-46%, 46-64% saturation with $(\text{NH}_4)_2\text{SO}_4$. The 0-25% fraction came down as a typical fibrin clot. The 25-46% and 46-64% fractions were filtered using Whatman No. 50 filter paper, and the precipitates were dissolved in water, these solutions showing thrombase activity. The results are summarized in Table VI.

The fractionation with $(\text{NH}_4)_2\text{SO}_4$ in some unknown manner produced thrombase. To eliminate the possibility of calcium ions in the activation, potassium oxalate was added to the prothrombase solution before fractionation in a final concentration of 0.2% potassium oxalate. The results are summarized in Table VII. In this experiment, there was no 0-25% fraction, most of the protein precipitated between 25 and 46% saturation. Approximately 0.4 mg. of protein N per cc. was lost in the fractionation due to the fibrinogen being coagulated (Table VI). The oxalate did

not inhibit the formation of thrombase. The activity found in both experiments was comparable. Calcium is therefore not the agent responsible for the activation produced by fractionation with (neutral) $(\text{NH}_4)_2\text{SO}_4$. These results on prothrombase are similar to those obtained by fractionation of thrombase with an acid saturated $(\text{NH}_4)_2\text{SO}_4$ solution (7).

TABLE VI

The Thrombase Activity of Solutions of the Precipitates Obtained from a Prothrombase (A) Solution by $(\text{NH}_4)_2\text{SO}_4$ at 0-25%, 25-46%, 46-64% Saturation (pH 7.0)

% Saturation	Units per cc.	mg. N* per cc.	Units per mg. N	Fraction
0-25†	—	—	—	
25-46	5	.50	10	I
46-64	5	.09	56	II

* Protein nitrogen determined by modification of method of Sørensen and Sørensen (7).

† Fraction came down as a fibrin clot.

TABLE VII

The Thrombase Activity of Solutions of the Precipitates Obtained from a Prothrombase (A) Solution Containing 0.2% $\text{K}_2\text{C}_2\text{O}_4$ by $(\text{NH}_4)_2\text{SO}_4$ at 0-25%, 25-46%, 46-64% Saturation (pH 7.0)

% Saturation	Units per cc.	mg. N* per cc.	Units per mg. N	Fraction
0-25	—	—	—	
25-46	5	.90	6	III
46-64	5	.07	71	IV

* Protein nitrogen determined by modification of method of Sørensen and Sørensen (7).

The fractions were titrated against precipitin sera for fibrinogen, pseudoglobulin, and thrombase. The results are summarized in Table VIII. The fractions reacted with the thrombase precipitin serum but none reacted with the pseudoglobulin precipitin serum. Activation also occurred without the presence of calcium ions and without splitting off all of the fibrinogen (Fractions III and IV). This experiment may indicate two different mechanisms in the activation of prothrombase to thrombase, the first due to calcium and the second due to a salt effect, both producing a splitting of the inactive prothrombase complex resulting in active thrombase. In the presence of calcium ions, the normal reaction takes place with the splitting off of fibrinogen and its conversion into fibrin. In the absence of calcium ions, the

TABLE VIII

Precipitin Reactions of Fractions Obtained by $(\text{NH}_4)_2\text{SO}_4$ Fractionation of Prothrombase (A) with Pseudoglobulin, Fibrinogen, and Thrombase Precipitin Sera

Fraction	mg. pro- tein N per cc.*	Normal rabbit serum	Serums of rabbits given injections of Pseudo- globulin	Fibrin- ogen	Thrombase (B)
I	.05	—	—	—	+
II	.009	—	—	—	+
III	.09	—	—	+	+
IV	.007	—	—	+	+

* 1-10 dilution of original fraction in 0.9% NaCl solution.

fibrinogen is not removed but the split occurs in such a manner as to liberate the active groups needed for thrombase activity. The fibrinogen in combination cannot be clotted; if it is split from the complex, fibrin will be formed. This is also true in prothrombase solutions which sometimes show slight thrombase activity. The inactive prothrombase molecule would appear to be activated by any agent that has the power of splitting fibrinogen from the complex and leaving the rest of the molecule intact.

DISCUSSION

Prothrombase produces precipitins to prothrombase, thrombase, fibrinogen, and euglobulin whereas thrombase produces precipitins to prothrombase, thrombase, and euglobulin. With the use of thrombase as an antigen, the precipitin production for fibrinogen is insignificant and indicates that fibrinogen is probably present as an impurity in the preparation. In four of the seven thrombase experiments, no precipitins to fibrinogen could be detected. One can conclude from these experiments that prothrombase is a fibrinogen-euglobulin-X complex which when activated to thrombase yields fibrinogen plus a euglobulin-X complex or thrombase. One can postulate that in Phase I of the blood clotting mechanism, prothrombase, a fibrinogen-euglobulin-X complex is converted into an active coagulating agent (thrombase) by splitting off the fibrinogen.

Bordet (17), Mellanby (18), Mills (19), Pickering (20) have suggested that the blood fibrinogen is either attached to prothrombase or acts as a stabilizer and that the activation of prothrombase is accomplished only after the fibrinogen is removed. Our results, in part, confirm the suggestions made by the above investigators.

It is interesting to note that the protein complex containing thrombase is precipitated between 46–64% saturation with $(\text{NH}_4)_2\text{SO}_4$ but gives precipitins to euglobulin when injected into rabbits. A true euglobulin is insoluble in water and precipitates below 46%. The thrombase complex is soluble in water, is precipitated above 46% and contains euglobulin. This can be explained because the protein combinations which make up prothrombase and thrombase do not have the same solubilities as the individual members of the complexes and have different precipitation limits with $(\text{NH}_4)_2\text{SO}_4$. Thrombase behaves like an albumin in its physical and chemical properties but immunologically it shows the reactions of euglobulin and perhaps some other protein. Since euglobulin will not clot oxalated plasma, it may be the carrier or stabilizer for the enzyme. The albumin, pseudoglobulin, euglobulin, and fibrinogen solutions used as antigens in this study were free from prothrombase and thrombase activities.

Eagle (21) and Eagle and Harris (22) found that certain proteolytic snake venoms and trypsin could be substituted for thromboplastin and calcium ions in the conversion of prothrombase to thrombase. Mellanby and Pratt (23), using fowl plasma, interpreted the clotting action of trypsin as being due to the liberation of thromboplastin from an inactive form in the plasma. Ferguson and Erickson (24) re-examined the question of trypsin clotting and believe that trypsin by proteolysis acts to make not only thromboplastin but also calcium available for clotting. These trypsin experiments indicate a proteolytic splitting off of fibrinogen from the fibrinogen-euglobulin-X complex liberating thrombase. Perhaps calcium ions and thromboplastin act in the same way by proteolysis.

SUMMARY

1. Beef prothrombase produces precipitins which react with prothrombase, thrombase, fibrinogen, and euglobulin. Beef thrombase produces precipitins which react with prothrombase, thrombase, and euglobulin. In three of the seven experiments, thrombase also produced weak, transitory precipitins to fibrinogen which would indicate that in all probability fibrinogen is present as an impurity rather than as an essential part of the complex. Prothrombase contains at least two plasma proteins: fibrinogen and euglobulin; thrombase contains euglobulin but not fibrinogen.

2. *In vitro* adsorption experiments will not differentiate between euglobulin and thrombase.

3. The antigen or antigens in thrombase are species specific.

4. Fractionation of prothrombase with (neutral) $(\text{NH}_4)_2\text{SO}_4$ produces thrombase. Calcium ions are not necessary in this reaction.

5. On the basis of these immunological and chemical studies, it is concluded that prothrombase is a fibrinogen-euglobulin-X complex and is converted into an active coagulating agent (thrombase) by splitting off the fibrinogen.

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Preparation of Prothrombin Products: Isolation of Prothrombin and Its Properties¹

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INTRODUCTION

Most attempts to concentrate prothrombin have been planned with the view of using the concentrate for the preparation and purification of thrombin. The work of Mellanby is an exception (1). He was primarily concerned with prothrombin itself and was able to describe a number of properties accurately, but the material which even he obtained was not sufficiently pure for most studies. For example, he came to the erroneous conclusion that prothrombin is insoluble in water. It is evident that if much progress is to be made in the elucidation of the blood clotting mechanism, prothrombin must be available in essentially pure form.

In 1937 work was begun with that objective in view. The process which was described (2) has been studied continuously since that time (3, 4). By introducing additional fractionation techniques we have now been able to obtain highly active preparations and believe that they represent the pure proenzyme itself.

In this paper techniques are described which can be used for the preparation of a number of prothrombin products, as well as pure prothrombin. These procedures are the result of a great many experiments which are not described. Specific details are outlined because experience has shown that any departure from the methods given is apt to result in poor yields, even though such modifications may seem reasonably promising. In most instances the description deviates only

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slightly from previously published methods, but the repetition is necessary to establish continuity for the innovations.

In the isolation procedure a prothrombin concentrate is first obtained by isoelectric precipitation from diluted plasma. This concentrate, together with a large mass of fibrinogen, is redissolved. $\text{Mg}(\text{OH})_2$ is then added to adsorb the prothrombin.² The adsorbed prothrombin is eluted by decomposing the $\text{Mg}(\text{OH})_2$ under pressure with CO_2 . The eluate is fractionated with $(\text{NH}_4)_2\text{SO}_4$ in the cold, and finally the prothrombin is separated by isoelectric precipitation.

GENERAL METHODS

Imidazole Buffer. 1.72 g. of imidazole (Eastman Kodak) were dissolved in 90 cc. of 0.1 N HCl, and diluted to 100 cc. with water. The pH of this isotonic buffer is 7.25 (5).

Oxalated Saline. 0.075 g. $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$ was mixed with 0.85 g. NaCl and dissolved in 100 cc. water.

Prothrombin Analysis. Two methods were used. One, the method of Warner, Brinkhous, and Smith (6), was used for plasma prothrombin assay and in most instances for prothrombin products. The other method, which we call the 3·1·1 set-up, was only used on fractions free of antithrombin. 0.3 cc. of neutral prothrombin solution were mixed with 0.1 cc. of purified lung extract (7), and 0.1 cc. of 0.6% CaCl_2 in saline. After an incubation period of 30 minutes the thrombin activity was determined by previously described techniques (8). The two-stage method gives the same analytical figures as the 3·1·1 method. The latter cannot be used when antithrombin is present. It should also be mentioned that the prothrombin in these products does not form thrombin as readily as native plasma prothrombin does, and consequently the incubation time must be extended in the original two-stage method. Extensive experience with all kinds of prothrombin fractions has convinced us that the two-stage method measures plasma prothrombin concentration accurately.

Beef Plasma. Beef blood was obtained at the slaughter house. One liter of special anticoagulant (4) (1.85% $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$ + 0.5% $\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) was placed in a 5 gallon pail and thoroughly mixed with 4.5 gallons of blood. As soon as possible the

² By varying the quantity of $\text{Mg}(\text{OH})_2$ used it is possible to govern yield and purity. Within limits a large amount of $\text{Mg}(\text{OH})_2$ will adsorb maximum quantities of prothrombin but many impurities will also be adsorbed. Conversely, small amounts of $\text{Mg}(\text{OH})_2$ will leave much prothrombin in solution, but the proportion of impurities in the final product is very considerably reduced. In either case, the presence of fibrinogen is highly desirable, because, next to prothrombin, it is most readily adsorbed. If prothrombin-like molecules are present, rather than the fibrinogen, their removal from the prothrombin eluates is not so simple. On the other hand the properties of fibrinogen are so strikingly different from those of prothrombin that its subsequent removal presents no special chemical problem.

plasma was obtained by centrifugation, frozen with the use of dry ice and alcohol and stored at -10° .

Mg(OH)₂ Cream. Slowly, and with stirring, 5 liters of concentrated NH_4OH were added to 20 liters of 20% MgCl_2 . The precipitate was allowed to settle, and was washed several times to remove ammonia. 500 g. of centrifuge packed Mg(OH)_2 were suspended in 1 liter of water.

Dialysis. Visking seamless cellulose tubing, 29/32 inch inflated diameter, was used (Visking Corporation, Chicago). To insure rapid dialysis the casing was only half filled and agitated mechanically with a vertical pull once every second. For the removal of $(\text{NH}_4)_2\text{SO}_4$ from refined products about 10 inches of casing length was allowed for each 10 cc. of solution to be dialyzed. With total immersion of the casing, a large membrane surface area was utilized for dialysis.

Solvent for Solubility Curve. A saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was first prepared at room temperature. 63 cc. of this solution were mixed with 7 cc. of imidazole buffer and 100 cc. of distilled water. This solvent was stored at 0° .

Tyrosine Analysis. The method of Folin and Ciocalteu (9) was used. 0.1 cc. of prothrombin was mixed with 0.5 cc. 10% NaOH and placed at 100° for 30 minutes. After cooling 7.4 cc. water, 1 cc. phenol reagent, and 3 cc. 20% Na_2CO_3 were added. After 30 minutes the color intensity was measured with a photoelectric colorimeter.

PROTHROMBIN PRODUCTS

The fractionation steps which have been studied in detail are outlined in Table I. These various products have certain characteristics which make them valuable for certain purposes. A few comments are therefore given. Product No. 4, for example, represents a refined product which invariably contains appreciable amounts of thromboplastin. By precipitating prothrombin from this product by addition of 1% HCl to pH 5.1 the purity can almost be doubled. The precipitate can be taken up in oxalated saline and neutralized. Usually the thromboplastin content will then be low, and the solution can be preserved at -40°C . for more than a year with very little loss of activity.

Product No. 10 is also very stable at -20° . It usually contains quantities of thromboplastin, is free of antithrombin, and while it is not as high a quality product, the prothrombin yield is exceptionally high.

Sulfanilamide-treated prothrombin, product No. 6, is a unique preparation which promises to be useful for theoretical studies concerned with stability. The sulfanilamide was added before the prothrombin eluate was dialyzed for the purpose of removing magnesium salts. As a consequence there is no loss of activity during the dialysis procedure. The prothrombin is very sensitive to such compounds as

oxalates, salicylates, and citrates, but not to 3,3'-methylenebis(4-hydroxycoumarin). These facts can be demonstrated most strikingly with oxalates. When, for example, product No. 5 is dried from the frozen state there is no loss of activity following reconstitution with water or saline. If the dried protein is dissolved in oxalated saline it is difficult to detect any appreciable amounts of prothrombin activity even if one proceeds with the prothrombin analysis promptly.

TABLE I
Preparation of Prothrombin Products

Procedure	Pro-thrombin product number	Total units	Units per mg. protein N
To 3 liters oxalated beef plasma add 42 liters cold distilled water, and mix with 1% acetic acid until acidified to pH 5.1. Allow to settle 4 hours. Decant. Collect the precipitate by centrifugation in the cold. Add 500 cc. cold oxalated saline (0.5% $K_2C_2O_4$ + 0.9% NaCl) and disperse with a Waring blender. Bring the pH to 6.4 with 0.1 N NaOH (approx. 30 cc.). Centrifuge in the cold to remove insoluble proteins. Proceed with next step at once. Dissolved acetic acid precipitate.....	1	6.0×10^5	18
Use prothrombin product No. 1 and stir with a mechanical stirrer. Add 140 cc. of cold $Mg(OH)_2$ suspension. Centrifuge cold in angle centrifuge. Discard supernatant liquid. Wash $Mg(OH)_2$ with 200 cc. cold water. Centrifuge in angle centrifuge. Repeat washing. Suspend $Mg(OH)_2$ in 250 cc. water, place in a glass pressure bottle and decompose the $Mg(OH)_2$ by shaking with CO_2 at 2-2.5 atmospheres pressure for 30 minutes. Stop shaking and after 10 minutes release CO_2 pressure. Prothrombin eluate...	2	4.1×10^5	215
Use prothrombin product No. 2. Allow to stand 1 hour. Strain through gauze to remove magnesium carbonate crystals and any fibrinous material. Clarified prothrombin eluate.....	3	4.1×10^5	1900
Use prothrombin product No. 3. Place in Visking casings and dialyze against repeated changes of cold distilled water until the specific resistance of the prothrombin solution is approximately 1500 ohms. The dialysis must be completed in less than 20 hours (over night). Strain to remove fibrinous material. Dialyzed prothrombin eluate.....	4	3.8×10^5	2000

TABLE I—*Continued*

Procedure	Pro-thrombin product number	Total units	Units per mg. protein N
Use prothrombin product No. 4 and dry from the frozen state. Purified prothrombin dried	5	3.8×10^5	2000
Use prothrombin product No. 2. Allow to stand 1 hour. Add 1 g. sulfanilamide. Strain through gauze to remove magnesium carbonate crystals, fibrinous material, and most of the undissolved sulfanilamide. Place in Visking casings and dialyze against repeated changes of cold distilled water until the specific resistance of the prothrombin solution is approximately 1600 ohms. The dialysis must be completed in less than 20 hours (over night). Strain to remove fibrinous material. Dry from the frozen state. Sulfanilamide treated prothrombin	6	4.1×10^5	2000
Use prothrombin product No. 2. Set at room temperature (26°) over night. Strain through gauze to remove magnesium carbonate crystals and fibrinogen gel. Clarified prothrombin eluate for $(\text{NH}_4)_2\text{SO}_4$ fractionation work	7	4.1×10^5	2500
Use prothrombin product No. 1 and stir mechanically. Add 200 cc. cold $\text{Mg}(\text{OH})_2$ suspension. Centrifuge cold in angle centrifuge. Discard supernatant. Wash $\text{Mg}(\text{OH})_2$ twice with 200 cc. cold water. Suspend $\text{Mg}(\text{OH})_2$ in 350 cc. water. Place in glass pressure bottle and decompose the $\text{Mg}(\text{OH})_2$ by shaking with CO_2 at 2–2.5 atmospheres pressure for 30 minutes. Stop shaking and after 10 minutes release CO_2 pressure. Cool 1 hour and strain through gauze. High yield prothrombin eluate	8	6.0×10^5	1000
Use prothrombin product No. 8. Dialyze over night against repeated changes of cold water. Dialyze until the specific resistance of the prothrombin solution exceeds 1600 ohms. Strain through gauze to remove fibrinous material. High yield dialyzed prothrombin eluate	9	5.0×10^5	1400
Use prothrombin product No. 9. Acidify to pH 5.1 with the use of 1% HCl. Collect the precipitate by centrifugation in the cold. Suspend the precipitate in 20 cc. oxalated saline and neutralize to pH 7.2 with the use of 0.1 N NaOH. High yield purified prothrombin	10	3.3×10^5	1800

ISOLATION OF PROTHROMBIN

For this work product 7 is used immediately after it has stood at room temperature over night. It usually has an activity of 2000 units per mg. nitrogen, and a volume of 300 cc. If the over-all yield is less than 65% the product is regarded as unsatisfactory for further work. Fig. 1 shows that it is not often necessary to discard the material. Most of the yields are over 65%.

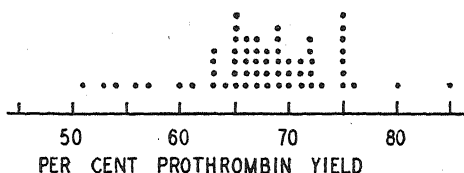


FIG. 1

Frequency Distribution of Prothrombin Yields for Prothrombin Product Number 7

Ammonium Sulfate Fractionation. Preparation number 727 was carried through as follows: Prothrombin product type No. 7 had a volume of 310 cc. Assay showed the prothrombin concentration to be 1290 units per cc. and, therefore, represented approximately a yield of 67%. The prothrombin solution was placed in a 1500 cc. beaker, set in a salt ice bath, and stirred. When it had reached a temperature of 0°, warm saturated (26°) $(\text{NH}_4)_2\text{SO}_4$ solution was added dropwise from a separatory funnel. Throughout this addition the temperature was kept at 0°. When 310 cc. of $(\text{NH}_4)_2\text{SO}_4$ had been added (50% of saturation) it was centrifuged 10 minutes in an angle centrifuge at 3500 r.p.m. Although the centrifuge was in a room refrigerated to -5° the solution warmed to about 2°. The clear supernatant solution was again cooled to 0°, and saturated $(\text{NH}_4)_2\text{SO}_4$ solution was again added dropwise until the concentration was 65% of saturation. After all the $(\text{NH}_4)_2\text{SO}_4$ had been added the temperature was allowed to drop to -5° (30 minutes). The solution was set in the -5° room for another 30 minutes. The prothrombin suspension was decanted from the inorganic crystals at the bottom of the beaker and centrifuged at 3500 r.p.m. The precipitate was collected in a single centrifuge tube and dissolved in 10 cc. of cold saline. The over-all prothrombin yield was 173,000 units or 29%. It is probable that this analytical result is

lower than the true figure, because $(\text{NH}_4)_2\text{SO}_4$ interferes with prothrombin analysis. The purity was found to be 13,500 units per mg. of tyrosine. Table II shows the results of other experiments carried through in the same manner. The $(\text{NH}_4)_2\text{SO}_4$ fractionated material

TABLE II
Fractionation of Prothrombin Product Number 7

Preparation number	Ammonium sulfate fractionation		Isoelectric fractionation	
	Yield per cent	Units per mg. tyrosine	Yield per cent	Units per mg. tyrosine
701	29	10,600	16	14,400
706	22	7,600	10	8,200
708	28	8,500	14	8,500
711	23	8,600	15	9,250
713	26	10,500	17	14,700
718	28	10,000	17	13,200
725	23	8,300	16	9,400
727	29	13,500	19	15,200
801	17	8,200	12	10,300
802	26	9,700	17	14,300

TABLE III
Properties of Prothrombin

Nitrogen.....	14.49%
Tyrosine.....	10.0%
Carbohydrate (oreinol).....	4.3%
Glucosamine.....	+
Tryptophan.....	+
Sulfur.....	+
Heat coagulation.....	—
With Ca^{++} alone.....	No thrombin
With Ca^{++} + Thromboplastin.....	100% thrombin
With Sr^{++} + Thromboplastin.....	80% thrombin
Stability.....	Over 2 weeks at 50°
Concentration in plasma (300 u./cc.).....	20 mg. %
Vitamin K.....	No evidence

loses all of its activity if dried from the frozen state and is not stable in solution. It is, therefore, necessary to proceed with the next step at once.

Isoelectric Fractionation. The above dissolved prothrombin solution was brought to pH 5.3 by adding 1% HCl with stirring so as to minimize local excess of acid. It was then dialyzed (*cf.* Methods) against

repeated changes of acidified cold distilled water. This cold (5°) distilled water was acidified to exactly pH 5.3 with dilute HCl. After 3 hours of dialysis all $(\text{NH}_4)_2\text{SO}_4$ had been removed. The prothrombin precipitate was collected by centrifugation, and dissolved in saline with the aid of 0.1 N NaOH. The activity was 15,200 units per mg. tyrosine and the total yield 17%. The yields in similar experiments are shown in Table II. We show the results of some less successful work for the purpose of bringing out the point that the isoelectric fractionation does not yield a high quality product if the $(\text{NH}_4)_2\text{SO}_4$ fraction has less than 9000 units per mg. tyrosine.

If there are no traces of $(\text{NH}_4)_2\text{SO}_4$ remaining, the prothrombin can be dried from the frozen state without loss of activity. Final drying is completed with the use of P_2O_5 and a vacuum desiccator. The prothrombin should be sealed in a glass tube or ampoule, since the protein will take on moisture rapidly.

PROPERTIES OF PROTHROMBIN

Analysis for Purity. Throughout this work the Tiselius electrophoresis apparatus was used for guidance. It was soon discovered that the active component migrates faster than the impurities, and at one time we hoped to separate the prothrombin by electrophoresis;

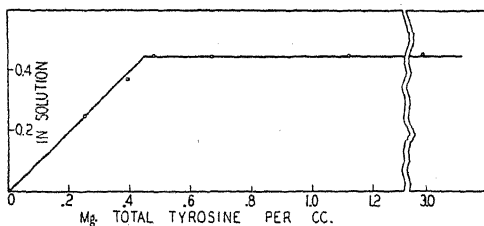


FIG. 2

Solubility Curve of Prothrombin with a Specific Activity of 15,200 Units per mg. Tyrosine

Solvent: 37% saturated ammonium sulfate, pH 7.6, temperature 0°.

but thus far we have never been able to maintain the activity throughout all the necessary manipulations. With the use of material possessing a specific activity of 12,000 units per mg. tyrosine, it was possible to predict that prothrombin could be expected to have a specific activity

of 13,000 to 15,000 units per mg. tyrosine (10). After preliminary work with solubility curves, preparation 727, which had a specific activity of 15,200 units per mg. tyrosine, was analyzed. The curve obtained (Fig. 2) indicates a single component. To obtain the data, the solid prothrombin was equilibrated with the $(\text{NH}_4)_2\text{SO}_4$ solution (pH 7.6, 37% of saturation) at 0° . The material which failed to pass into solution was removed by centrifugation at 0° .

Activation with Ca^{++} (cf. Table III). Optimum amounts of Ca^{++} were added to a neutral solution of prothrombin. The mixture was tested for thrombin activity during the next four hours. No thrombin was formed; it all remained as prothrombin.

Activation with Ca^{++} and Thromboplastin. A neutral solution was mixed with optimum amounts of Ca^{++} and a large excess of purified lung extract. Within one minute the thrombin titer was equivalent to that predicted for the product on the basis of the two-stage analytical procedure for prothrombin. It remained there for many hours, showing that no antithrombin was present.

Activation with Sr^{++} and Thromboplastin. A large excess of thromboplastin was mixed with the neutral prothrombin solution. The Sr^{++} concentration was varied systematically. At all concentrations conversion was slow, and at optimum concentration only 80% of the thrombin obtainable with calcium was found in the reaction mixture.

Stability. The dry product, in sealed tubes, was placed in an oven at 50° . At the end of two weeks no loss of activity could be detected. At the end of two months there was a loss of approximately 30%.

Concentration in Plasma. The activity of prothrombin is 1500 units per mg. dry weight (ash free basis). In human plasma its concentration is approximately 300 units per cc. It can, therefore, be calculated that the plasma concentration is 20 mg. %. If we consider the plasma volume as 5% of body weight, a 60 kilogram man will have 600 mg. of prothrombin in his circulation. This assumes that the specific activity is the same from one species to another.

Heat Coagulation. The protein solutions are not coagulated by heating at pH 7.

Sulfur. Qualitative tests were positive.

Tryptophan. The Hopkins-Cole reaction was positive. The intensity of the red color suggests the presence of considerable quantities.

Nitrogen, Tyrosine, Carbohydrate. The material was dialyzed to remove sodium chloride, dried from the frozen state, and vacuum

desiccated over P_2O_5 . The nitrogen content (Kjeldahl) was 14.49%. Tyrosine (*cf.* Methods above) 10.0%.

Carbohydrate was estimated by the method of Tillmans and Phillipi (11) as modified by Sørensen and Haugaard (12). Colorimetric measurements were made with a Beckman spectrophotometer, using equal quantities of galactose and mannose as standard. The orcinol reaction indicates the presence of 4.3% carbohydrate. In addition, qualitative tests show that glucosamine is present in considerable quantity. If there is one molecule each of galactose, mannose, and glucosamine, the total carbohydrate content is nearly 6%.

Ultraviolet Absorption Curve. The absorption curve for prothrombin in acid and alkaline solution is shown in Fig. 3. There is no evidence

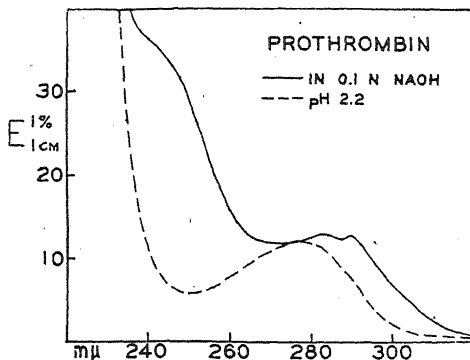


FIG. 3

Ultraviolet Absorption Curves of Prothrombin in Acid and Alkaline Solution

for the presence of vitamin K. If it is present it must be in minute quantities.

The shape of the curves and the changes in shape associated with variations in pH indicate that the absorption can be ascribed to the tyrosine and tryptophan in the molecule. In fact, solutions made up to contain equal quantities of tryptophan and tyrosine give absorption curves which are very similar to those of prothrombin. It seems likely that the tryptophan content of the molecule is at least as high and perhaps higher than in most plasma proteins.

Two Prothrombins. Quick (13, 14, 15) has postulated the existence of two prothrombins. In our work we have encountered no evidence

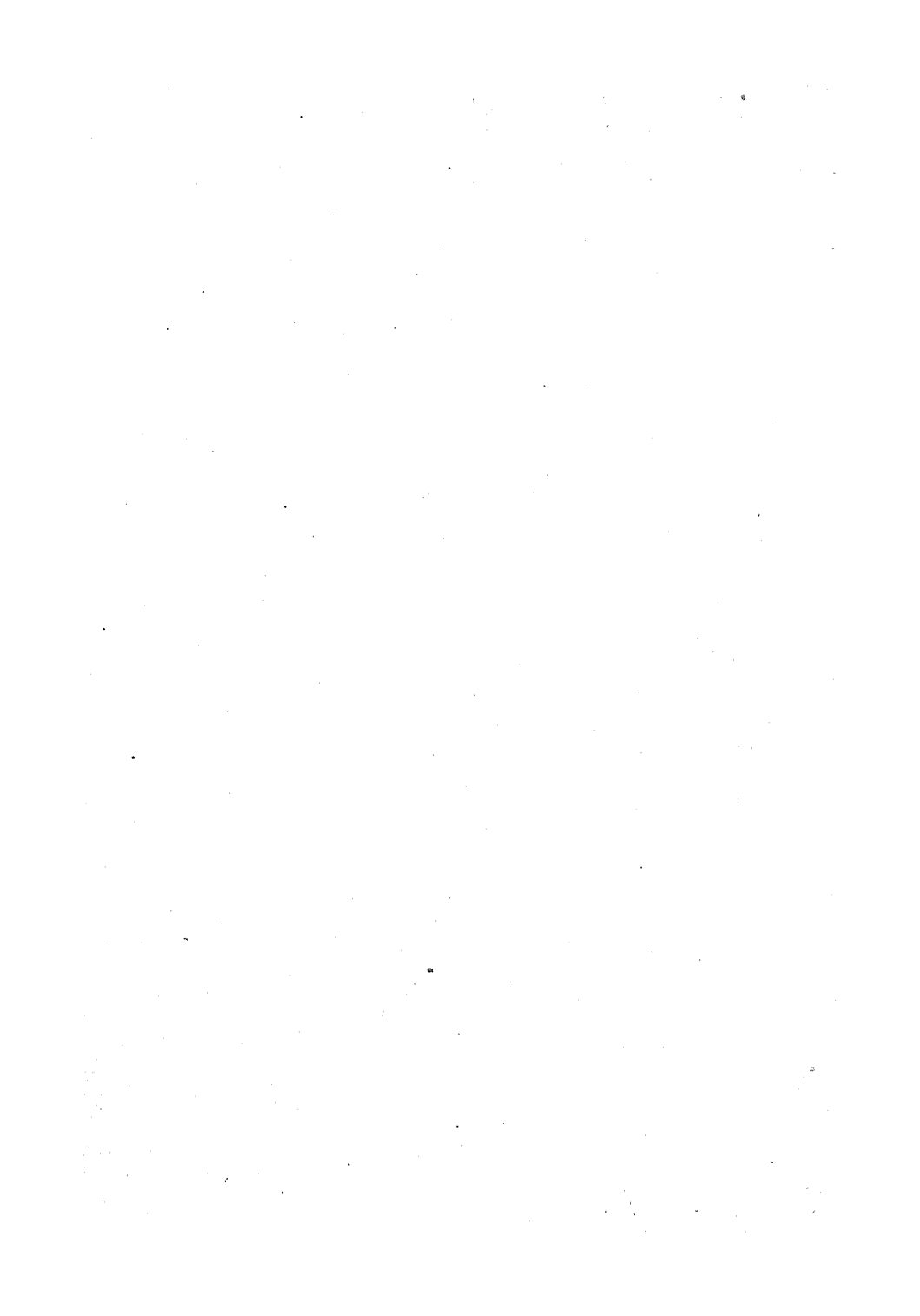
to substantiate his view. His conclusion is based on evidence obtained with the use of complicated native systems, only one type of assay technique was employed, and furthermore, plasmas from different species were mixed. It, therefore, seems to us that his experiments will eventually be reinterpreted.

SUMMARY

The preparation of several antithrombin-free prothrombin products and their properties have been described. One of these products has been analyzed with the use of the solubility curve technique. It was composed of a single component, and, therefore, is regarded as representing pure prothrombin. This prothrombin is a snow white, amorphous, water-soluble glycoprotein. On the basis of the orcinol reaction, the carbohydrate content is 4.3%, and in addition glucosamine is present. On an ash free basis, the specific activity is 1500 units (Iowa) per mg. dry weight. By calculation it is deduced that plasma prothrombin concentration is 20 mg. % (300 units per cc.). Calcium alone does not activate prothrombin. Strontium can be substituted for calcium but complete conversion to thrombin does not take place. Tests for tryptophan and sulfur are positive. Micro-Kjeldahl analysis indicates 14.49% nitrogen; and the phenol reagent of Folin indicates the presence of 10.0% tyrosine. The ultraviolet absorption curve for prothrombin can practically be duplicated in the region above 240 m μ with a mixture of tyrosine and tryptophan. This work shows no evidence for the existence of more than one prothrombin.

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A Modification of the Thiochrome Method for the Rapid Determination of Thiamine in Urine *

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INTRODUCTION

In search of a more rapid procedure than that afforded by the existing methods for the determination of thiamine in the urine which would be suitable for large numbers of daily analyses involved in human nutrition surveys, the following method was evolved. With its aid it is possible for one well-trained analyst to carry out 16 determinations in a working day.

The method consists essentially of the following steps: adsorption of the thiamine on superfiltrol as proposed by Emmett, Peacock and Brown (1); elution with an acid pyridine-methanol solution, elaborated in this study; and the oxidation, extraction and fluorometric reading by the technique described by Mason and Williams (2), including their sulfite blank provision.

Reagents

Superfiltrol. "For thiamine determinations," as furnished by The Filtrol Corporation, 315 West 5th Street, Los Angeles, California.

Sodium Sulfite, Na_2SO_3 . 5% solution, prepared daily.

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Eluting Solution. Merck's Reagent pyridine, kept over charcoal, is filtered as needed; to 200 ml. are added 800 ml. of water and 200–210 ml. of concentrated HCl, adjusting the solution approximately to pH 1, followed by 600 ml. of Merck's Reagent methanol.

Alkaline Ferricyanide. 150 ± 5 mg. of powdered K_3FeCN_6 is dissolved in 0.5 ml. of water, followed by 30 ml. of 10 *N* NaOH. This solution is made up every 4 hours.

Standard Solutions. A stock solution containing 0.100 mg. thiamine chloride per ml. in 0.05 *N* HCl is kept in an amber bottle in the refrigerator. A working standard solution containing 1 γ per ml. is prepared weekly from the stock solution, also in 0.05 *N* HCl, and is kept in the refrigerator.

0.5 M Acetate Buffer pH 4.6. Prepared by mixing equal volumes of 0.5 *N* acetic acid and 0.5 *N* sodium acetate solution.

25% KCl Solution. 250 g. of the salt dissolved in and made up to 1 liter with water.

n-Butanol. As furnished in 5 gallon cans by Commercial Solvents Corporation, is satisfactory without redistillation.

Sodium Sulfate, Na_2SO_4 . Merck's Dried Powder, N.F.

Apparatus

Centrifuge Tubes. 15 ml. graduated, Pyrex.

Separatory Funnels. Squibb, glass stoppered, 125 ml. Kimble Glass Co., preferably supported in wooden racks holding 6 funnels each. The stopcocks are lubricated with a non-fluorescent mixture consisting of 3 parts by weight of beef tallow melted and mixed with 1 part of mineral oil. (Greases containing vaseline should not be used.) The new Dow Corning Silicone stopcock grease was found to be satisfactory.

An Angle Centrifuge. With head for six 15 ml. tubes, such as Clay Adams No. CT-1000X.

A Fluorometer. With filters for B_1 determinations; Coleman Photofluorometer Model 12 is satisfactory.

PROCEDURE

An aliquot, 1–5 ml., of urine expected to contain 0.5–2 γ of B_1 , previously adjusted to pH 4.0–4.5, is measured into each of three 15 ml. graduated centrifuge tubes. To tube B ("Blank") 0.5 ml. of a fresh 5% Na_2SO_3 solution and water to 5 ml. are added; the tube is then immersed for 20 minutes into boiling water and cooled. To tube R ("Recovery") 1.00 ml. of a standard solution, containing 1 γ of B_1 , and water to 5 ml. are added. To tube X ("Unknown") (urine only) water is added to the 5 ml. mark. To all 3 tubes are added 1 ml. of 0.5 *M* acetate pH 4.6 buffer and 0.2 g. Superfiltrol, which may be conveniently measured in a leveled small porcelain spoon. The contents of the tubes, closed with rubber stoppers, are mixed by slow back-and-forth inversion by hand for 3 minutes (the tubes may be conveniently held together in a beaker for this purpose). The stoppers

and sides of the tubes are rinsed with water and the volume brought up to 11 ml.; the tubes are then centrifuged for 3 minutes in an angle centrifuge. The clear supernatant fluids are decanted and discarded. The Superfiltrol is washed with about 6 ml. of water by shaking as above, rinsing sides of tubes and stoppers, bringing volume up to 11 ml.; centrifuging, and decanting. To the sediment the elution mixture is added to the 6.1 ml. mark. The tubes are stoppered and shaken again by inversion for 3 minutes, and centrifuged, the eluates being decanted for use.

The oxidation is carried out as follows: Into each of three 125 ml. glass stoppered Squibb funnels arranged in a rack are pipetted 9 ml. of 25% KCl solution, followed by 3.00 ml. (one-half of the original urine sample taken for analysis) of the three eluates. This is followed by 12 ml. of normal butanol.* A brisk air current is started through the three mixtures (or through one at a time) by means of pipettes attached to a compressed air line (or to a tank of nitrogen). Two ml. of a freshly prepared alkaline ferricyanide solution are blown in rapidly from a wide tipped pipette with the aid of a rubber bulb. The aeration is continued for 60 seconds and stopped by removing the air-tube. The butanol and water layers separate well in a few minutes, while the next oxidations are carried out. The water layer is drained off, and the butanol is cleared by the addition (from a spoon) of 1 g. of Na_2SO_4 (Merck's Dried Powder) and by gentle shaking. The butanol layer, which must be absolutely clear, is decanted through the mouth of the funnel into a fluorometer tube or cuvette. The readings are made in a fluorometer equipped with the usual filters for the estimation of thiochrome.

The calculations are

Readings of Urine — "Blank" = x divisions

Readings of "Recovery" — "Unknown" = y divisions due to 0.5γ of B_1

$\frac{x}{2y} = \gamma$ of B_1 in aliquot taken for oxidation.

DISCUSSION

Extensive and intensive experience with photometric methods involving the measurement of small concentrations of thiochrome in

* Less than 12 ml. of solvent may be used if the smaller size fluorometer cuvettes or tubes are available, thus increasing the sensitivity of the method accordingly.

relatively large amounts of urine has convinced us that with each sample of urine it is necessary to determine its own blank and recovery of standard values. This point is best illustrated in the data shown in Table I. It is clear that blank values may differ with varying volumes

TABLE I

Variability of "Blank" and "Recovery" Values in Duplicate Determinations with Varying Aliquots of Human Urines

These represent 1 hour's excretion after an oral dose of 5 mg. thiamine hydrochloride.

Subject	Total volume <i>ml.</i>	Sample analyzed <i>ml.</i>	Urine U	Blank B	U-B	Recovery div./ γ	Calculation	Total B ₁ γ
			Galvanometer divisions					
22	50	0.5	15	10	5	56	$\frac{5}{56} \times \frac{50}{0.5} = 9$	
		1.5	30	17	13	54	$\frac{13}{54} \times \frac{50}{1.5} = 8$	
32B	50	0.5	70	14	56	50	$\frac{56}{50} \times \frac{50}{0.5} = 112$	
		0.25	41	10	31	56	$\frac{31}{56} \times \frac{50}{0.25} = 111$	
20A 1st hr.	130	0.5	12	9	3	56	$\frac{3}{56} \times \frac{130}{0.5} = 14$	
		1.5	17	9	8	54	$\frac{8}{54} \times \frac{130}{1.5} = 13$	
20A 2nd hr.	550	1.5	15	9	6	60	$\frac{6}{60} \times \frac{550}{1.5} = 37$	
		2.5	20	10	10	60	$\frac{10}{60} \times \frac{550}{2.50} = 37$	

of the same urine taken for analysis and also in different urines; in this group of values the range is from 9 to 17 galvanometer divisions. The "recovery" values also vary with the volume of the aliquot and with each urine sample; in this group the range is from 50 to 60 divisions per 1 γ B₁. Actually much wider variations in both the "blank" and "recovery" values have been observed. In spite of these wide

fluctuations, excellent agreement of values can be obtained for the thiamine content of urine samples, not only in exact duplicates, but also when varying amounts of urine are taken for analysis.

The reproducibility of values is further illustrated in Table II. Urines collected from patients in 3 periods covering 4, 6, and 14 hours were analyzed separately and also the total 24-hour output as obtained by pooling aliquots of the above 3 samples. The sum of the values of

TABLE II
Agreement in Patients' Urines When Analyzed for Thiamine in 3 Separate Periods and in Pooled 24 Hour Urines

Subjects			McR. Control	A.R. Urines, I	H.B.	L.S.	M.R.	E.M.
Period		Hrs.	γ	γ	γ	γ	γ	γ
1	8 a.m.—12	4	5	100	12	24	4	3
2	12 — 6	6	22	95	19	14	31	4
3	6 — 8 a.m.	14	24	132	43	82	35	15
I	Sum (1+2+3)	24	51	327	74	120	70	22
I	Pooled	24	67	332	75	125	74	22
Urines II, after 1 mg. B ₁ intramuscularly at 8 a.m. and 500 mg. nicotinamide orally at 6 p.m.								
4	8 a.m.—12	4	73	130	41	134	122	25
5	12 — 6	6	141	172	41	104	22	4
6	6 — 8 a.m.	14	17	66	52	67	62	41
II	Sum (4+5+6)	24	231	368	134	305	206	70
II	Pooled	24	237	374	136	300	192	69

the 3 separate analyses agrees well with the value obtained on the pooled 24-hour urine. In this series, urine No. 6 in each case was collected during the 14 hours following the administration of 500 mg. of nicotinamide as a saturation test (5). These urines contained from 40 to 180 γ *N*¹-methylnicotinamide per ml. It has been found by various investigators that in larger concentrations this substance interferes with the thiochrome method. Najjar and Ketron (4) have recently proposed a correction. We have found that under the conditions used in our procedure the "blank" compensated for the interference quite satisfactorily. This is borne out by the data in Table II and also by other results obtained with urines upon the addition of 100–500 γ *N*¹-methylnicotinamide per ml. of urine. These findings corroborate those of Mason and Williams (2).

It may be recorded, incidentally, that 95 to 100% of the *N*¹-methyl-nicotinamide added, or as found present in the urine by analysis, was recovered in the acid pyridine-methanol eluates. These eluates may therefore be used for the analysis of both thiamine and *N*¹-methyl-nicotinamide. This should be particularly useful in those cases in which interfering substances cause an unusually high blank value in the direct determination of *N*¹-methyl-nicotinamide as described by Huff and Perlzweig (3).

The reproducibility of results shown in Tables I and II was only achieved after the most scrupulous standardization of the technique and thorough training of each individual analyst in the details of the procedure. Until each analyst standardizes his technique by repeated practice with triplicate and quadruplicate determinations, preferably using varying aliquots of the same urine, the results are quite erratic and show considerable deviations, particularly in urines with low concentrations of thiamine. Several days of practice in learning to perform the various operations in a standardized and uniform manner is therefore absolutely essential.

The steps in the procedure which require special attention are:

1. The simultaneous and identical treatment of the 3 tubes containing the "blank," "unknown," and "recovery" of any given urine sample in regard to amount of Superfiltrol taken; mode and period of agitation during adsorption and elution; centrifugation and washing; the mode of addition of the alkaline ferricyanide, aeration, etc.

2. The standardization in the use of the photofluorometer. The steadiness of galvanometer readings must be ascertained. Differences of 2 divisions or more in several consecutive readings on the same solution may result in errors of $\pm 25\%$ as may be readily seen from the data in Table I, especially in urines of low thiamine concentration. This standardization may be conveniently tested beforehand by means of a standard solution of quinine sulfate. The cuvettes or tubes should be standardized in the fluorometer with the above quinine solution for uniformity. We found it necessary to place a permanent mark on that portion of the tube which is to face the source of light in order to give a uniform reading. The exposed portion of tubes should be covered completely from extraneous light during the reading. The surfaces of the tubes are carefully cleaned with soft cloth or paper, and even slight turbidity of the butanol extracts is to be scrupulously avoided for obvious reasons.

The fluorescence intensity of the butanol extracts is remarkably stable; it remains constant for at least 18 hours, and is unaffected by daylight.

The use of the above described method during the past year has yielded a body of data on the urinary excretion of thiamine by well-fed and mal-nourished human subjects, before and after known doses of thiamine. The range of values obtained coincides well with that reported in the recent literature. Detailed data on the application of this method to a tolerance test in a large group of individuals on normal and subnormal intakes will be published separately.

The procedure described in this paper has been thus far applied to human urine only. Its applicability to urines of other animal species is yet to be established.

SUMMARY

A rapid fluorometric thiochrome method for the determination of thiamine in human urine has been developed. It is based on the adsorption technique of Emmett, Peacock, and Brown (1); elution with an acid pyridine-methanol solution; and the sulfite blank, oxidation and extraction procedures described by Mason and Williams (2). It has been demonstrated that it is necessary for each sample of urine analyzed to be calculated in terms of its own "blank" and "recovery" of added thiamine values.

After a few days of preliminary training aimed at standardization of the various steps, an experienced analyst can carry out 16 determinations in a working day, with a good degree of accuracy and reproducibility.

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Partial Purification of Histaminase *

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INTRODUCTION

First attempts to purify histaminase were made by McHenry and Gavin (1). They succeeded in obtaining a preparation with 20 times more activity than minced hog kidney. They assayed biologically the histamine left after incubation with histaminase. Kiese (2) used repeated precipitation with ammonium sulfate for his purification, but apparently was not as successful as McHenry and Gavin. Laskowski (3) attempted to purify histaminase, using oxygen consumption as a test for this enzyme. He found, however, that in the crude preparations the amount and the rate of oxygen consumption were influenced by the presence of heme compounds, therefore the degree of purification calculated on this basis was not considered reliable and was omitted.

More recently Stephenson (4) has described a preparation having 20-33 times more activity than the original tissues (hog kidney). His technique was identical with that of Kiese, only repeated fractionations with ammonium sulfate being used.

The present work describes a method leading to some further purification of histaminase. Both oxygen consumption and ammonia production were used for determination of activity.

METHODS

The Warburg apparatus was used for the determination of the oxygen consumed. The conditions of the experiment were the same as those described in a previous paper (3).

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Ammonia production was determined by a modified Conway method. Instead of Conway vessels, small screw-top bottles were used. 0.1 ml. of 0.0088 *N* H_2SO_4 containing 0.025% of bromo-thymol blue, was pipetted into a flat-bottom vial of about 2 ml. capacity, and the vial was placed within the bottle. Buffer and enzyme solutions were then pipetted into the bottles, and the bottles were placed in the incubator for at least 15 minutes to reach the temperature prior to the addition of histamine. The enzymic reaction was stopped by addition of 1 ml. of saturated potassium carbonate solution. The bottles were further incubated for at least six hours, usually overnight. The vial was removed, and the remaining acid was titrated within the same vial with 0.00587 *N* NaOH ; 1 ml. of NaOH being equivalent to 100 μg . of NH_3 . When tested with known amounts of ammonium sulfate varying from 20 to 120 μg . the average error was $\pm 5\%$.

EXPERIMENTAL

It was shown in the previous paper (3) that histaminase, after being purified from heme-containing compounds, consumed one atom of oxygen per mol of histamine. This finding was confirmed. After the heated enzyme had been purified by fractionation with ammonium sulfate (second step of purification), only one atom of oxygen per mol of histamine was used in the histamine-histaminase reaction. The enzyme preparations were tested after each step during further purification, and it was found that oxygen consumption persisted on the level of one atom per molecule. This is also in agreement with the conclusions of Kiese (2) and of Stephenson (4).

In order to find out the applicability of the test based on the ammonia production a few experiments were performed. The test was found of no value in the case of the crude extract of kidney cortex. This was due to enormous production of ammonia by the extract itself, a finding already emphasized by Kiese (2). Heating to 62° considerably decreased this spontaneous NH_3 production, but the blanks were still very high.

For the purpose of investigating the method, the normal course of purification was changed (see preparation). The first salt fractionation was carried out with sodium sulfate instead of ammonium sulfate. The fraction obtained between 30 and 60% saturation contained most of the histaminase and showed only small blanks. These blanks were apparently the result of the hydrolysis occurring after the addition of K_2CO_3 and could have been easily taken into account. Similar results have been secured with preparations fractionated with $(\text{NH}_4)_2\text{SO}_4$ after prolonged dialysis.

At this stage of purification 1 mol of ammonia was split from 1 mol of histamine, Fig. 1. The imidazole ring was not affected, as shown by the diazo reaction. Fairly good proportionality was found between the amount of enzyme and the amount of ammonia produced (pro-

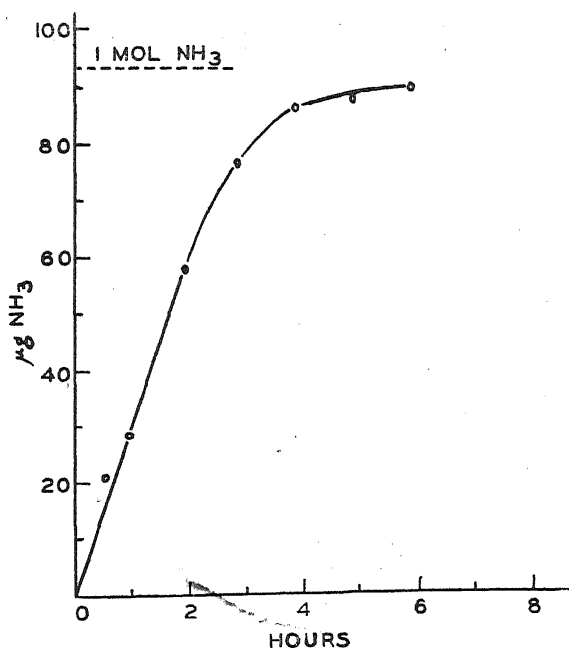


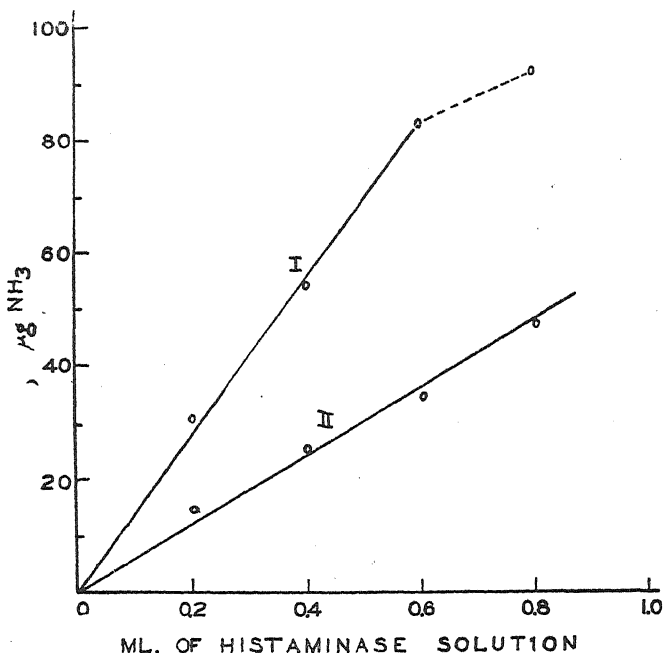
FIG. 1

Production of Ammonia during Histamine-Histaminase Reaction

1 mg. histamine dihydrochloride
1.0 ml. histaminase (3.5 mg. protein)
pH 7.2, air, $t = 38^{\circ}$.

vided the substrate was in excess). This is shown in Fig. 2 and in Table I. When the enzyme was the limiting factor the excess of the substrate did not influence the results to any considerable degree (Table I).

The results of these experiments justify the use of ammonia determination as a test for the histaminase activity, unfortunately only during later stages of purification. The method, however, is laborious and slow and requires a considerable amount of enzyme for the series



Effect of Different Concentrations of Histaminase Solution on the Amount of NH_3 Produced
 Curve I. Histaminase solution (3.5 mg. protein/ml.). Incubation time 4 hours.
 Other conditions as usual.
 Curve II. Different preparation of histaminase, contains 2 mg. protein/ml.
 Incubation 6 hours.

TABLE I
 Effect of the Concentration of Histamine on Ammonia Production
 Histaminase: 3.5 mg. protein per ml. Incubation 6 hours

Histamine dihydrochloride mg.	NH ₃ produced	
	0.2 ml. Histaminase μg.	0.8 ml. Histaminase μg.
1.0	28	92
2.0	29	118
3.0	29	114

of experiments. In practice, from the second step on, purification was followed by the oxygen consumption method and only the successful steps were verified by the ammonia production method. The agreement between the two methods was satisfactory.

The activity of histaminase was expressed in arbitrary units, one unit being equivalent to the amount of enzyme, which under the experimental conditions (total volume of the liquid 3 ml., 1 mg. histamine dihydrochloride, pH 7.2, 38°, air) will utilize 1 μ l. of oxygen per minute. It will roughly correspond to the production of 1.5 μ g. of NH_3 per minute, and to the destruction of 1 mg. of histamine dihydrochloride per hour. The degree of purification of the enzyme was expressed as a number of units per mg. of protein. Protein was determined by the method of Robinson and Hogden (5).

PREPARATION OF HISTAMINASE

Extraction. Hog kidneys were obtained from the local slaughterhouse as fresh as possible. The medullas were removed and rejected. Three kg. of kidney cortex were ground (in portions) in the Waring blender with 9 liters of 1% NaCl. The mixture was heated to 62° with mechanical stirring. It was centrifuged in an ordinary centrifuge. Usually between 6 and 7 liters of red fluid were collected. The precipitate was rejected.

No way was found to express the activity of the enzyme at this stage, because neither oxygen nor ammonia methods were reliable. Judging by analogy with more purified histaminase, it was considered that no appreciable loss of activity occurred at this stage. This conclusion was also supported by the fact that the yield of enzyme obtained from the heated and unheated preparations, after subsequent precipitation with ammonium sulfate was slightly higher in the heated.

Precipitation with Ammonium Sulfate. Crude extract was treated with 151 g. of solid ammonium sulfate per liter. A small, flocculent precipitate was filtered off and discarded. The filtration was always slow and filters had to be changed frequently. 209 g. of solid ammonium sulfate were added per liter of filtrate (to make 50% saturation), the yellow-brown precipitate was collected, and the red filtrate containing most of the hemoglobin was discarded. The precipitate was redissolved in about 1 liter of water. It was tested for dry weight and protein content, and the content of salt calculated from the difference. Solid ammonium sulfate then was added to make 50% saturation. The final precipitate was collected and redissolved in about 300 ml. of water. At this stage the average preparation contained 0.15 units per mg. of protein. The average yield was about 1800 units. Probably 15–20% of activity was lost during this stage, but the degree of purity was at least doubled.

Adsorption on Calcium Phosphate and Elution. A suspension of $\text{Ca}_3(\text{PO}_4)_2$ containing approximately 0.1 mol per liter was prepared by mixing 1 liter of 0.4 M Na_3PO_4 with 1 liter of 0.6 M CaCl_2 and adjusting the pH to 7.0. The gel was washed 5–7

times with distilled water before being used. Approximately 50–75 ml. of calcium phosphate suspension were used for each gram of protein in the histaminase solution. After centrifugation, the supernatant liquid was tested for activity. If it still contained more than 10 per cent activity, more $\text{Ca}_3(\text{PO}_4)_2$ was added. The final adsorption complex was centrifuged down. It was first eluted with 500 ml. of *M*/15 phosphate buffer of pH 7.2. This elution showed little activity and was discarded. The second elution was made with an equal volume of the same buffer to which 20 g. of sodium citrate had been added, to make a 4 per cent solution in respect to sodium citrate. The centrifugation of this elution in the ordinary centrifuge never produced a clear supernatant. The usual procedure was to centrifuge 2–3 hours. The opalescent eluate was then treated with 60 g. of sodium citrate per 100 ml.

The precipitate which formed contained all the activity, and almost all protein. It was filtered, suspended in about 100 ml. of water and dialysed against 1% NaCl in the cold. At this stage the average preparation contained 0.3 units per mg. of protein, and the average yield was about 900 units.

Second Heating to 65°. The dialysed liquid was heated to 65° with mechanical stirring. The precipitate was centrifuged off and discarded. No significant loss of activity occurred at this stage and the average degree of purification was usually doubled. The supernatant was treated as below.

Fractional Precipitation with Sodium Sulfate. For each 100 ml. of histaminase solution 13.5 g. of anhydrous sodium sulfate was added, and the precipitate was filtered off and discarded. To the filtrate an additional 13.5 g. of Na_2SO_4 were added, the mixture was heated to 37° and filtered in the incubator. The precipitate¹ was redissolved in

TABLE II
Degree of Purification Achieved at Different Stages

Stages of purification	Degree of purification—units per mg. of protein				
	Exp. 21	Exp. 22	Exp. 23	Exp. 24	Average
2. After fractionation with $(\text{NH}_4)_2\text{SO}_4$	0.12	0.25	0.20	0.21	0.19; 0.15*
3. After elution	0.24	0.58	1.2†	0.44	0.45; 0.30*
4. After second heating	0.51	1.0	0.9	1.0	0.85
5. After fractionation with Na_2SO_4	0.85	1.2	2.9	1.7	1.66

* These two figures represent averages from 15 preparations; other figures in this column are averages from the 4 preparations listed in the table.

† This figure was not averaged.

¹ At this stage the preparation can be kept for a period of 3 weeks without detectable loss of activity, when stored under a small volume of saturated sodium sulfate in the refrigerator.

TABLE III

Relationship between the Amount of Histamine, Oxygen Consumption and Ammonia Production after Completion of the Histamine-Histaminase Reaction

Histamine dihydrochloride mg.	Oxygen consumed		Ammonia produced	
	found μl.	calculated μl.	found μg.	calculated μg.
0.2	11.9	12.1	20.0	18.6
0.5	33.6	30.3	48.0	46.5
1.0	61.7	60.5	89.0	93.0

a minimal amount of water and dialysed against 1 per cent NaCl in the cold. At this stage the average degree of purification was 1.5 units per mg. of protein, and the average yield was 450 units.

Table II summarizes the results of the purification obtained at the different stages. Table III shows relationship between the amount of histamine, oxygen consumed and ammonia produced when the purified enzyme was used.

DISCUSSION

In spite of the fact that several units of histaminase have already been proposed (1, 4, 6), a newly defined unit has been used during this work. The advantage of this new unit is the ease with which it may be calculated from either oxygen or ammonia methods. By definition the new unit is about 5.4 times larger than the unit described by Zeller (6), about 10 times larger than the unit described by Stephenson (4), and approximately 24 times larger than the "Torantil unit" of the Winthrop Chemical Company.

It is not possible to calculate exactly the degree of purification achieved by the described procedure, because no reliable figure was obtained for the activity of the minced tissue. Rough approximation could be made on the following assumptions: (1) Assuming that the minced tissue utilizes on the average 2 atoms of oxygen per mol of histamine, we found that one kilogram of minced kidney (approximately 200 g. of protein) contained between 400 and 700 units of histaminase. Similar figure for initial histaminase content will be obtained on the assumption that extraction of histaminase was complete, and that during the first heating no loss of activity occurred. The maximal yield ever obtained in a second stage of purification was 700 units from 1 kg. Therefore original kidney cortex could have contained a maximum of $1000/200,000 = 0.005$ unit per mg. of pro-

tein; comparing this with the average degree of purity of the finished preparation (1.5), a 300-fold concentration had been achieved. (2) According to Stephenson kidney cortex contains 150 (Stephenson's) units per gram of protein. Considering our unit as approximately equal to 10 Stephenson's units, our average final preparation will have 15 Stephenson's units per mg. of protein. In such case concentration has been 100-fold. Comparing values reported by Stephenson for his purified histaminase our preparation is about 5 to 7 times more active. Through the courtesy of Winthrop Chemical Company, we were able to compare our preparation with their preparation T. 360-N, which according to the specification contains 5 Torantil units per ampule, which will roughly correspond to $5/24$ of our unit. We found 29 mg. of protein per ampule. Expressed in our units it would contain 0.007 unit per mg. of protein. When tested according to our procedure T. 360-N gave the same result.

No definite statement as to the nature of the histaminase can be made. Zeller (7) considered it to be a flavo-protein. He claimed to have identified flavin after splitting the enzyme with acetone. We were unable to confirm his findings using our preparation. Attempts were also made to split histaminase according to the method of Warburg and Christian (8). The results were negative. If the pH dropped below 4 no activity could be recovered in either fraction, or in both recombined. If the pH was kept higher, part of the activity was present in the precipitate, but no increase in activity was observed on addition of the supernatant or on addition of flavin-adenine dinucleotide from yeast. If histaminase is a flavo-protein its protein moiety must be more unstable towards acid than that of other flavo-proteins, and its prosthetic group must be bound more firmly.

The question of whether or not H_2O_2 is formed during the reaction also can not be answered by direct evidence. Histaminase prepared according to the present method always contained catalase, in amounts sufficient to destroy postulated amounts of H_2O_2 .

SUMMARY

A method for the partial purification of histaminase has been described. By this method an average yield of 450 units of histaminase was obtained from 3 kg. of hog kidney cortex. The average activity was 1.5 units per mg. of protein.

Partially purified histaminase utilizes 1 atom of oxygen and produces 1 mol of ammonia per mol of histamine.

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The Action of Histaminase in Vivo *

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INTRODUCTION

Since the discovery of histaminase several attempts have been made to demonstrate its activity *in vivo*. All these attempts, however, were made with entirely insufficient amounts of histaminase. It is obvious that in order to show any action of histaminase within the living animal the amount employed must be large enough to destroy at least half of the active dose of histamine within the time between introduction of histamine and the onset of the symptoms.

There are many contradictory statements concerning the action of histaminase *in vivo*. Fairly recently positive results were reported by Karady and Browne (1). Their shocking dose was 4 mg. of histamine (free base), injected intraperitoneally into 250 g. guinea pigs. Karady and Browne stated their protective dose of histaminase as 3 ampules of Torantil (T 360)² injected intravenously. Assuming the potency of Torantil did not change from the time of testing to the time of the experiment, it contained 3 Winthrop units, one Winthrop unit being equivalent to the amount of histaminase which will destroy 1 mg. of histamine in 24 hours. Assuming further, only for the sake of argu-

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¹ Some of the data included in this report were taken from the thesis submitted by Janet M. Lemley to the Graduate School of the University of Arkansas in partial fulfillment of the requirements for the degree of Master of Science.

² Torantil was also available in ampules containing 5 units each (T 360-N). Should Karady and Browne have used a stronger preparation the active dose after 30 minutes in the control and experimental animals would be represented by 4 mg. and 3.69 mg. of histamine respectively. The difference still would not have accounted for more than 10% of the lethal dose.

ment, that the histamine has been absorbed immediately, that the dilution of the substrate and of the enzyme by the total volume of the blood had no effect on the velocity of the reaction, and that a straight proportionality exists between the time and the amount of the substrate destroyed—the amount of histaminase used in these experiments could have destroyed 3 mg. of histamine within 24 hours, or $3/48 = 0.06$ mg. within 30 minutes. At the end of that time the effective dose in control and experimental animals would be represented by 4 mg. and 3.94 mg. of histamine respectively. In spite of this Karady and Browne reported protection in more than 75% of their animals. Alexander and Bottom (2) and Youngner, Freedman and Nungester (3) were unable to confirm the results of Karady and Browne.

In the present work experiments were undertaken which were similar to the above except that the amount of histaminase employed was greatly increased.

MATERIALS AND METHODS

Guinea pigs were used as experimental animals because they are sensitive and uniform toward histamine. A smaller animal would have been a better one, but rats are notoriously resistant to histamine shock, and no data were available for the golden hamster in this respect.

The dose of histamine was fixed as one and a half times the lethal dose as listed in Sollman and Hanzlik "Fundamentals" (4). Histamine dihydrochloride (Eastman) was used. For the intraperitoneal injections 6 mg. of histamine dihydrochloride were used, usually for a guinea pig around 300 g. For the intracardial or intravenous injection 1.6 mg. of histamine dihydrochloride were used per kg. of body weight.

Intravenous injections were given into the external jugular vein which was exposed by skin incision over the area under procaine, local anesthesia. Intracardial injections were done without anesthetic.

Histaminase was prepared according to the method described in a previous paper (5). Only preparations containing at least one unit (see previous paper) per mg. of protein were used. Histaminase was administered in physiological saline; the volume never exceeded 3 ml. Histaminase was always injected into the blood stream either intracardially or intravenously immediately preceding the administration of histamine.

RESULTS AND DISCUSSION

The results of the experiments are shown in Tables I–III. No animal of the first control group survived longer than 30 minutes. In the second experimental group of 13 guinea pigs 6 of the animals survived longer and therefore were at least temporarily protected. Four animals

TABLE I

Control Animals, No Histaminase

Date and no. of animal	Histamine dihydrochloride injected		Time of survival	No. animals dead within 30 minutes	No. animals surviving longer than 30 minutes
	amount	route			
Jan. 10 No. 1	6 mg.	intraperitoneal	min. 21	1	0
Jan. 10 No. 2	6 mg.	"	20	1	0
March 9 No. 3	6 mg.	"	21	1	0
March 16 No. 4	6 mg.	"	20	1	0
April 1 No. 5	6 mg.	"	12	1	0
April 3 No. 6	1.6 mg./1 kg.	intracardial	5	1	0
April 3 No. 7	1.6 mg./1 kg.	"	4	1	0
April 3 No. 8	6 mg.	intraperitoneal	22	1	0
May 9 No. 9	6 mg.	"	20	1	0
July 1 No. 10	6 mg.	"	29	1	0
Total 10				10	0

TABLE II

Experimental Animals, Histamine Injection Immediately Following Injection of Histaminase

Date and no. of animal	Histaminase injected		Histamine dihydrochloride		Time of survival	No. of animals dead within 30 min.	No. of animals surviving longer than 30 min.	No. of animals surviving longer than 3 days
	dose	route	dose	route				
Jan. 10 No. 1	150	intracardial	6 mg.	intraperitoneal	∞	0	1	1
March 9 No. 2	100	"	6 mg.	"	∞	0	1	1
March 9 No. 3	200	"	6 mg.	"	37 min.	?	?	0
March 16 No. 4	100	"	6 mg.	"	>7<16 hours	0	1	0
April 1 No. 5	100	"	6 mg.	"	21 min.	1	0	0
April 1 No. 6	100	"	6 mg.	"	19 min.	1	0	0
April 1 No. 7	100	"	6 mg.	"	17 min.	1	0	0
April 3 No. 8	60	"	1.6 mg./1 kg.	intracardial	∞	0	1	1
April 3 No. 9	100	"	1.6 mg./1 kg.	"	2 min.	1	0	0
April 3 No. 10	100	"	1.6 mg./1 kg.	"	4 min.	1	0	0
April 3 No. 11	100	"	1.6 mg./1 kg.	"	13 min.	1	0	0
April 9 No. 12	120	intravenous	6 mg.	intraperitoneal	24 hrs.	0	1	0
July 1 No. 13	150	intracardial	6 mg.	"	∞	0	1	1
Total 13						6	6	4

TABLE III

Control Animals, Third Group, Histaminase Only

Date and no. of animal	Histaminase injected		Time of survival	No. of animals dead within 30 minutes	No. of animals surviving longer than 30 minutes	No. of animals surviving longer than 3 days (indefinitely)
	dose	route				
May 9 No. 1	100	intracardial	36 hours	0	1	0
May 9 No. 2	100	"	24 hours	0	1	0
June 23 No. 3	120	"	10 minutes	1	0	0
June 23 No. 4	120	"	∞	0	1	1
Total 4				1	3	1

survived indefinitely. All but one animal of the experimental group which died showed on autopsy heavy intrapericardial hemorrhage.

In the control group, where the animals received intracardially an equal amount of saline instead of histaminase, only two showed significant intrapericardial hemorrhage. A very characteristic picture was shown by two animals from the experimental group (Nos. 4 and 12 in Table II), which survived for several hours. They exhibited light symptoms of histamine poisoning during the first 30 minutes, appeared to recover from it, then instead of improving became gradually worse and died in coma.

The third group of animals was injected with histaminase only. Out of 4 animals only one survived indefinitely. Three others exhibited symptoms very similar to those seen in unsuccessfully protected animals from the second group. The autopsy findings were also similar.

The following conclusion was drawn: histaminase, at the present state of purity, is still highly toxic when injected into animals.

The hope that histaminase might be used as a tool for the investigation of anaphylaxis must be postponed until less toxic and more potent histaminase preparations can be secured. In order to obtain a fairly safe protection against $1\frac{1}{2}$ times lethal dose of histamine, the amount of histaminase should be increased up to 200 units or more, which is impossible with the present preparation.

SUMMARY

The action of histaminase *in vivo* has been shown in protecting at least $\frac{1}{3}$ of the guinea pigs, which simultaneously received $1\frac{1}{2}$ times the lethal dose of histamine.

Histaminase at the present state of purity is toxic, and the dose can not be increased to the level which will give a safe protection.

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Enzymatic Studies on Tissues of Rats Fed Purified Rations Containing Succinylsulfathiazole *

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INTRODUCTION

The fact that both pantothenic acid and biotin deficiencies produced a decreased rate of pyruvate metabolism in the liver (1, 2) suggested that this effect might be related to a single deficiency. Numerous investigations (3-10) have shown that an external source of one or more nutritional factors in addition to biotin (6, 8, 11-13), vitamin K (13-15), vitamin E (16), and possibly inositol (17), is required by rats when sulfasuxidine (succinylsulfathiazole) or sulfaguanidine (sulfanilylguanidine) is incorporated in a purified ration, presumably because of a decreased rate of synthesis of factors by intestinal bacteria (3-5, 11, 13, 15, 18, 19). Many investigators (6, 8, 10, 11, 13, 14, 21, 22) have demonstrated that one of these factors is the norite eluate factor of Snell and Peterson (20), usually designated as "folic acid." Wright and Welch have also indicated that folic acid and biotin are necessary for the *utilization* of pantothenic acid (21, 22). Furthermore, it seemed possible that a pantothenic acid or biotin deficiency could cause a decreased rate of synthesis or utilization of this or a related factor. An attempt was made to determine whether this might be the case by feeding rats a purified diet containing the known vitamins and sulfasuxidine.

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This report deals with the changes in activity of some of the respiratory enzymes of the liver and heart of rats fed sulfasuxidine, with the efficacy of various substances in counteracting these changes, and with the cause of the inhibition.

EXPERIMENTAL

Treatment of Animals. Twenty one-day-old rats of the Sprague-Dawley strain were placed on the following purified ration: sucrose, 72.5; casein (Labco, vitamin-free), 18; salts IV (23), 4; corn oil, 5; and sulfasuxidine, 0.5. Each rat was given the following vitamin supplement daily in one ml. of ten per cent alcohol: thiamine hydrochloride, 20 μ g.; pyridoxin hydrochloride, 25 μ g.; nicotinic acid, 25 μ g.; riboflavin, 30 μ g.; calcium pantothenate, 200 μ g.; biotin (as S.M.A. concentrate No. 1000), 0.5 μ g.; and choline hydrochloride, 10 mg. Once a week each rat received three drops of a 1:6 dilution of haliver oil containing 25 μ g. per drop of 2-methyl-1,4-naphthoquinone. On this regimen some animals exhibited a partial, spontaneous recovery. Therefore, in a second series of rats, the sulfasuxidine was increased to a level of one per cent. Although this level was effective in preventing the recovery, it also produced a high incidence of mortality. As a result, a level of 0.75% was chosen for most of the experiments.

The supplementary substances were given along with the B-vitamin solution. These were started during the second to eighth week of the experiment and were continued for periods of one to five weeks, until the rats were used for the enzymatic studies. These special supplements included the following, given daily: solubilized liver (Wilson "Fraction L"), 0.2 g.; 1:20 liver powder (Wilson's), 0.2 g.; folic acid concentrate prepared from grass juice powder (24) and autoclaved at pH 7.0 for one and one-half hours at fifteen pounds pressure, equivalent to 0.2 g. of solubilized liver as assayed by *Streptococcus lactis* (25); 2.0 mg. of inositol; 2.0 mg. of *p*-aminobenzoic acid; also the latter two together; 2.0 mg. of ascorbic acid; the folic acid concentrate autoclaved in 1 *N* HCl for one hour, equivalent before autoclaving to 0.2 g. of solubilized liver; see Ransone and Elvehjem (8). This preparation had no activity for *S. lactis* after acid autoclaving.

Studies of Enzymatic Activity. Measurements of oxygen consumption in the presence of various substrates were made in the Warburg respirometer at 37.5°C., in either 2.0 or 3.0 ml. of sodium phosphate buffer, pH 7.4, with 0.2 ml. of ten per cent KOH and a strip of filter paper in the center well to absorb the carbon dioxide. Readings were taken every ten minutes for thirty or forty minutes after a ten minute equilibration period. The values used were the maximum values observed during the course of the measurements; these usually occurred during the first ten-minute interval.

The substrates, pyruvate, fumarate, and lactate were prepared like those described in a previous paper (1). Sodium succinate (Merck), malic acid (Eastman), and glutamic acid [purified as described in Organic Syntheses (26)] were adjusted to pH 7.2-7.4. The assays for succinoxidase and cytochrome oxidase were performed essentially as described by Potter (27), and Potter and Schneider (28). No correction was made for the degree of homogenization of the tissue in the cytochrome

oxidase assay. The malic oxidase assay is that recently devised (29). The concentrations and amounts of reagents and tissue homogenates (30) employed are summarized in Table I. The tissue used in the pyruvate, fumarate, and lactate systems

TABLE I
*Constituents Used for the Assays of the Enzymes in the Tissues of Rats
Fed Sulfasuxidine*

Constituent	System				
	Pyruvate ml.	Fumarate ¹ ml.	Succinate ml.	Malate ml.	Cytochrome oxidase ml.
Sodium phosphate, 0.25 M, pH 7.4	0.3	0.3	0.4	0.4	0.27
Pyruvate, 0.20 M	0.4				
Fumarate, ¹ 0.30 M		0.3			
Succinate, 0.50 M			0.3		
Malate, 0.50 M				0.2	
Glutamate, 0.50 M				0.2	
Nicotinamide, 0.10 M		0.2		0.3	
Coenzyme 1, ^{2,3} 3.0 mg./ml.		0.1		0.5	
Cytochrome c, 4×10^{-4} M			0.1	0.1	0.4
CaCl ₂ , 0.012 M			0.1		
AlCl ₃ , 0.012 M			0.1		0.1
Sodium ascorbate, ^{3,4} 10.0 mg./ml.					0.5
2% Liver or 1/2% Heart homogenate in H ₂ O			0.5	1.0	0.1-0.2
10% Liver homogenate in buffer ⁵	1.0	1.0			
Water, q.s.	3.0	3.0	3.0	3.0	2.0

The final pH in all flasks was 7.4 to 7.5.

¹ In the lactate system, an equivalent amount of lactate is substituted for fumarate.

² Coenzyme 1 (Merck), about 50% pure.

³ Added from the side arm during equilibration.

⁴ Prepared immediately before use; adjusted carefully to pH 6.9-7.1.

⁵ 0.017 M sodium phosphate, 0.13 M sodium chloride, pH 7.4.

was homogenized in a cold saline-phosphate buffer (0.13 M NaCl and 0.017 M sodium phosphate) of pH 7.4. The tissues employed in the succinate, malate, and cytochrome oxidase systems were homogenized in cold re-distilled water.

RESULTS

The livers from rats on the basal sulfasuxidine diet showed rates of pyruvate and fumarate oxidation only 20 to 40% of those for livers from normal rats. This decrease could not be overcome *in vitro* by the addition of coenzyme 1 or cytochrome c in either system, by the addition of magnesium ions or cocarboxylase in the pyruvate system, or

by any combination of these materials. The addition of 0.4 ml. of a "kochsaft," prepared by boiling one part of homogenized liver in three parts of water, was ineffective in increasing the rate of respiration. On the other hand, when rats that had been on the basal ration for seven to ten weeks were fed 0.2 g. per day of solubilized liver or 1:20 powder for one to two weeks, the growth inhibition was completely overcome and the enzymatic activity of the pyruvate and fumarate systems fell within the normal range.

It seemed unlikely that a single dietary deficiency would decrease the activity of several enzymes so markedly, especially when one considers the changes in enzyme activities found in other vitamin and mineral deficiencies (31, 32). Furthermore, there have been several reports of toxicities of various types in experimental animals fed sulfonamides. Chamelin and Funk (33) have shown that liver extracts decrease the toxicity of sulfonamides.

We therefore looked for some inhibitory substance in the livers of rats fed the drug. The contents of the flasks from the respiration studies were centrifuged to remove the tissue, and one ml. of the supernatant fluid was added to flasks containing normally respiring tissue. The respiration in the presence of pyruvate or fumarate was inhibited to the extent of 50 to 80% even though the extract represented one-third of the amount of tissue used in the respiration measurements; *i.e.*, it was a one to three dilution. No inhibition was observed when the extract was from normal liver. The same results were obtained when the livers were digested with pancreatin. Livers of rats receiving sulfasuxidine plus solubilized liver or 1:20 powder had normal enzymatic activity; extracts from those livers did not inhibit the enzymatic activity of normal liver. None of the extracts or digests caused any inhibition of the succinoxidase system.

These preliminary results necessitated further studies to determine: (a) the effect on other enzyme systems in the rat tissues; (b) the time required for this inhibition to appear in the animal; (c) the efficacy of various nutritional or detoxifying substances in counteracting the inhibition *in vivo*. Consequently, the second series of animals was divided into two groups: those on the basal drug ration and those on the same ration without sulfasuxidine. Starting on the tenth day, we made enzymatic studies on the livers and hearts of several rats from each group each week. There was no consistent lowering of respiration rate in the group on the drug until the sixth to seventh week of

experiment. It is at about this time that the rats develop severe symptoms of a deficiency or toxicity, including a plateau in growth rate.

The enzymatic determinations were extended to include the succinoxidase, malic oxidase, and cytochrome oxidase systems. These systems occasionally, but not consistently, fell below the normal values. Of 26 animals studied, seven succinoxidase values were between 15 and 65, and nine cytochrome oxidase values were from 105 to 180. The normal Q_{O_2} values are 70–110 and 200–400, respectively. The malic oxidase values followed a similar trend; the normal values are 50–80 with our assay method. These changes were distributed randomly among the various groups. The decreases in the succinoxidase and malic oxidase activities correlated with the decrease in the cytochrome oxidase activity. The lowered activity of the succinoxidase and the malic oxidase systems was partially overcome by increasing the cytochrome *c* to six times the concentration ordinarily used in these systems, *i.e.*, to the level used in the cytochrome oxidase assay to obtain the maximum activity of this enzyme. It appears that the decreased activities of these two systems are caused by the low cytochrome oxidase activity, and that one can attach no significance to the changes unless they can be shown to occur independently of the cytochrome oxidase system.

The need for folic acid and biotin for the utilization of pantothenic acid (21, 22) suggested the possibility of a decreased rate of utilization of nutritional factors other than those present in liver preparations. Schultze (32) has shown that the cytochrome oxidase activity is decreased in the liver and heart of rats deficient in copper. We therefore studied the activity of this enzyme in the heart as well as in the liver. The activity of the cytochrome oxidase in the heart was not altered. Furthermore, feeding 100 μ g. of copper per day plus that in the ration (2.5 μ g. per g.) did not cause the cytochrome oxidase in the liver to return to normal.

The effects of 2.0 mg. each, per day, of *p*-aminobenzoic acid and inositol together were variable. A substance that either indirectly or directly caused a growth response usually resulted in a counteraction of the toxicity.

Because of this variation, the level of the drug was increased to 0.75% for the last series of rats, and they were maintained on the basal ration for at least four weeks. They were then divided into groups of three rats each and supplemented with the substances listed under

Treatment of Animals. The ascorbic acid was included because it is a detoxifying agent under certain conditions (34). The period of supplementation and representative results of the enzymatic studies are summarized in Table II. In this last series of animals, the only sub-

TABLE II

Representative Results of Enzyme Activities of Livers of Rats Fed Sulfasuxidine

Group	Weeks on experiment	Weeks supplemented*	No. of assays	Pyruvate		No. of assays	Fumarate	
				QO ₂ †	Total activity‡		QO ₂ †	Total activity‡
1	6-11	—	12	2.2 (1.0-4.6)	11.1 (5.5-18.0)	4	2.6 (1.4-4.4)	13.4 (5.2-19.0)
2	8-11	1-2	5	9.2 (8.8-9.8)	36.3 (31.2-46.1)	2	12.4 (10.2-14.5)	52.5 (51.2-53.7)
3	9	2	2	9.3 (9.2-9.4)	37.3 (33.1-41.5)	2	8.7 (8.6-8.8)	34.8 (31.6-38.0)
4	7-8	4-6	4	2.6 (1.5-4.2)	15.0 (9.0-25.1)	4	5.2 (2.1-6.9)	30.5 (13.0-40.7)
5	7-11	7-11	8	9.8 (8.5-11.1)	37.8 (26.5-52.5)	8	10.4 (8.4-12.4)	40.0 (31.0-60.5)
6	6-12	—	—	8-12	—	—	9-16	—

Group 1, basal sulfasuxidine ration; Group 2, solubilized liver, 0.2 g./day; Group 3, 1:20 powder, 0.2 g./day; Group 4, acid-autoclaved folic acid, equivalent to 0.2 g./day of solubilized liver *before autoclaving*, no activity for *S. lactis* after autoclaving; Group 5, folic acid or neutral-autoclaved folic acid, given from the beginning of the experiment, equivalent to 0.2 g./day of solubilized liver, as assayed by *S. lactis*; Group 6, normal control and stock rats receiving no drug and no special supplements.

* Continued on sulfasuxidine ration during period of supplementation.

† QO₂ is the μ l. of O₂ consumed per mg. of dry tissue per hour.

‡ Total activity is the QO₂ times the per cent liver in the rat.

stances that effectively and consistently overcame the liver toxicity and the growth inhibition were solubilized liver and 1:20 powder. In earlier experiments, a folic acid concentrate fed from the beginning of the experiment gave good growth¹ and a normal respiration rate of the pyruvate and fumarate systems in the liver.

Although neutral autoclaving does not destroy "folic acid" (8), such a preparation, when given to four rats for three weeks after they had

¹ These rats were obtained from the studies reported by Ransone and Elvehjem (8).

been on the basal sulfasuxidine ration for eight weeks, produced only a small growth response which was delayed for the first week in three of the rats, and counteracted the toxicity in only two of the rats. The concentrate may be less potent than solubilized liver in factors not assayed by *Streptococcus lactis*, and hence a greater amount or a longer time of supplementation may be required for it to be fully effective. The enzymatic activities of the tissues of animals receiving copper, ascorbic acid, *p*-aminobenzoic acid, inositol, or acid-autoclaved folic acid for four to seven weeks were similar to those of the rats on the basal drug diet.

Most of the livers were analyzed for moisture and calcium. Those from rats on the basal sulfasuxidine ration were somewhat higher in moisture and contained from five to ten times up to fifty to one hundred times the amount of calcium in normal liver. In subsequent experiments, to be reported later, the decrease in pyruvate oxidase activity correlated well with an increased calcium content of the livers of rats fed sulfasuxidine or sulfathiazole. When solubilized liver was administered as a therapeutic measure to rats receiving these drugs or when sulfadiazine was fed, both the calcium content and the rate of pyruvate oxidation were normal.

DISCUSSION

The changes observed in the succinoxidase, malic oxidase, and the cytochrome oxidase systems can be attributed to the hepatic injury or degeneration, and not necessarily to any specific effect of the deficiency on the enzymatic activity, because the changes do not occur consistently in any one group. This interpretation is strengthened by the fact that the "total activity" of these enzymes of the liver is not decreased in half of those livers having lower Q_{O_2} values; i.e., the Q_{O_2} value multiplied by the per cent liver in the animal is comparable to that in the normal rats (see Axelrod, *et al.*, 35) because these rats generally have livers that are larger than those of normal rats. The oxidation in the presence of lactate was neither markedly nor consistently reduced. If there is any decrease *per se* in the enzymes metabolizing pyruvate or fumarate it is masked by the inhibiting substance which accumulates in the liver.

The inhibitor appeared to be one of two types of substances; (a) sulfasuxidine, the product of its hydrolysis, sulfathiazole, or some other decomposition product of sulfasuxidine; (b) a normal metabolite

which is toxic or inhibitory in high concentrations. Subsequent work in this laboratory has shown that the substance is not sulfasuxidine or sulfathiazole because neither sulfasuxidine, sulfathiazole, nor sulfapyridine inhibits the enzymes oxidizing pyruvate, fumarate, succinate, or malate (36). The fact that the toxic substance in the liver inhibits coenzyme-1-linked systems (Potter (37) has recently indicated that pyruvate oxidation requires coenzyme 1) and is stable to various treatments suggested that it is calcium.

SUMMARY

1. The enzymes metabolizing pyruvate and fumarate in the livers of rats fed purified rations containing sulfasuxidine (succinylsulfathiazole) have only 20 to 40% of the normal activity.

2. Feeding solubilized liver for one to two weeks after the rats have been on the basal ration for seven to nine weeks causes these systems to return to normal activity and reverses the growth inhibition.

3. Evidence is presented to indicate that the decrease in activity is caused by an inhibitory substance accumulating in the liver.

4. The decreases in succinoxidase, malic oxidase, and cytochrome oxidase noted in some animals can be attributed to the hepatic injury occurring in these animals.

5. No enzymatic change can be attributed directly to a nutritional deficiency on the basis of the experiments reported here.

6. The properties of the inhibitor suggested, and subsequent analysis proved that it was an excess of calcium.

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Further Studies on Tyrosinase in Aerobic Plant Respiration

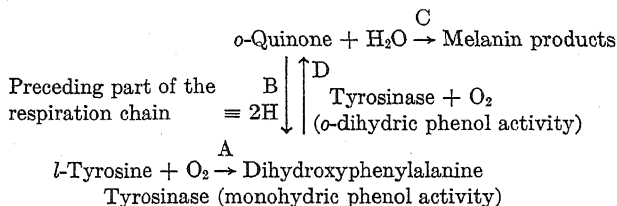
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INTRODUCTION

Boswell and Whiting (1) and later Baker and Nelson (2) have shown that tyrosinase plays the role of a terminal oxidase in the respiration of slices of the potato tuber (*Solanum tuberosum*). Recently Robinson and Nelson (3) showed that *l*-tyrosine, which occurs in the free condition in the tubers, constitutes the precursor of 3,4-dihydroxyphenylalanine and that the latter functions as the hydrogen carrier adjacent to the terminal oxidase in this respiration chain. They suggested the following series of reactions, indicated below, as being involved in the oxygen end of the chain.



The activity of tyrosinase varies towards different phenolic compounds, and is probably due to the respective affinities of these compounds for the enzyme (4). The tendency of the dihydroxyphenylalanine to combine with tyrosinase is so great, compared to that of tyrosine, that as soon as a small amount of the latter has been oxidized to the dihydroxy-compound (Reaction A) the concentration of the dihydroxy-compound becomes sufficient to prevent any more tyrosine from being oxidized. As the dihydroxyphenylalanine is oxidized to the

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corresponding *o*-quinone (Reaction D) the quinone is immediately reduced back to the dihydroxy form by reaction B. In other words, as long as the preceding part of the respiration chain is capable of returning the quinone back to the dihydroxy form Reaction C does not take place. Any disorganization of the preceding part of the respiration chain, such as bruising the plant and crushing the cells, so that the quinone is no longer reduced, causes Reaction C to take place. The darkening of peeled potatoes when exposed to air can be cited as an instance due to Reaction C.

The above view of how tyrosinase functions as the terminal oxidase in respiring potato slices is in line with the observations made by Robinson and Nelson (3). They found on adding tyrosinase, which is inactive towards ascorbic acid, to a mixture of tyrosine and ascorbic acid, practically no tyrosine was oxidized as long as any unoxidized ascorbic acid remained in the reaction mixture. The ascorbic acid played the same role in Reaction B as the preceding part of the respiration chain and permitted a small amount of dihydroxyphenylalanine, shuttling back and forth between the dihydroxy and quinone forms, to engage the enzyme to such an extent that all but a trace of the tyrosine remained unoxidized. However, as soon as all the ascorbic acid had been oxidized, Reaction B was interrupted, and the tyrosine then was oxidized at the same rate as when oxidized by the enzyme in the absence of ascorbic acid, and the reaction mixture became colored due to the accumulation of the products formed in Reaction C.

When Baker and Nelson added some *p*-cresol to respiring potato slices, they found that the rate of oxygen uptake was increased, whereas the rate of carbon dioxide evolved was not, and the reaction mixture gradually became colored. This observation is different from that of Robinson and Nelson, when they added *l*-tyrosine to respiring potato slices. The latter investigators found that the addition of tyrosine not only increased the rate of oxygen uptake but also that of the carbon dioxide given off, and that the R.Q. was close to unity. This difference in the behaviors of the two monohydric phenols can be accounted for by assuming *p*-cresol to have a greater affinity than tyrosine for the enzyme, an assumption which appears justified by the fact that *p*-cresol is oxidized by tyrosinase much faster than is *l*-tyrosine. The greater affinity of the *p*-cresol for the enzyme permits such a large amount of *p*-cresol to combine with the enzyme and become oxidized, that the reducing action in Reaction B is no longer capable of reducing

the quinone as fast as it is being formed in Reaction D. As a consequence less carbon dioxide would be formed than oxygen consumed. Furthermore, due to Reaction B being slower than D, some quinone would not be reduced, and therefore would enter into Reaction C, causing the reaction mixture to become colored as observed by Baker and Nelson.

Since sweet potato (*Ipomoea batatas* Poir) cells also contain tyrosinase, it was decided to study their respiration in the presence and absence of added monohydric phenols. Thin slices of these tubers were prepared as described by Boswell and Whiting (1) and the rates of oxygen uptake and carbon dioxide given off were measured in a Warburg respirometer. As pointed out above, the addition of *p*-cresol to respiring slices of the common potato only increased the rate of oxygen uptake, showing that this monohydric phenol can not take part in the respiration of the potato cells. However, it was found that in the case of the sweet potato slices, the *p*-cresol can take part in the cell respiration. Thus, as shown in Fig. 1, not only did the *p*-cresol increase the rate of oxygen uptake but also the rate of carbon dioxide given off and the latter to such an extent that the R.Q. continued close to unity. The homocatechol formed in the oxidation of the *p*-cresol functioned in Reactions B and D as a hydrogen carrier, by shuttling back and forth between its dihydroxy and quinone forms. The latter conclusion is supported not only by the fact that during the experiment no coloration of the reaction mixture in the respirometer flask was observed, but also by the fact that the difference between the total volumes of oxygen consumed in the two reaction vessels, the one containing the respiring slices and one mg. of *p*-cresol and the other (control) only the same number of slices but no added *p*-cresol, was 488 mm.³ (Fig. 2). Since the volume of oxygen necessary to oxidize 1 mg. of *p*-cresol to its final oxidized state (hydroxyquinone; i.e., Reaction A through Reaction C) is only 310 mm.³, it follows that at least 178 mm.³ of oxygen must have been consumed in taking care of the hydrogen or its equivalent, furnished by the preceding part of the respiration chain in Reaction B. Furthermore, since no coloration occurred in the reaction mixture, only a small portion of the *p*-cresol was oxidized in the presence of the sweet potato slices. Most of it, like the tyrosine in the presence of the slices of the common potato, observed by Robinson and Nelson, remained unoxidized and served as a reservoir for the formation of homocatechol, the hydrogen carrier

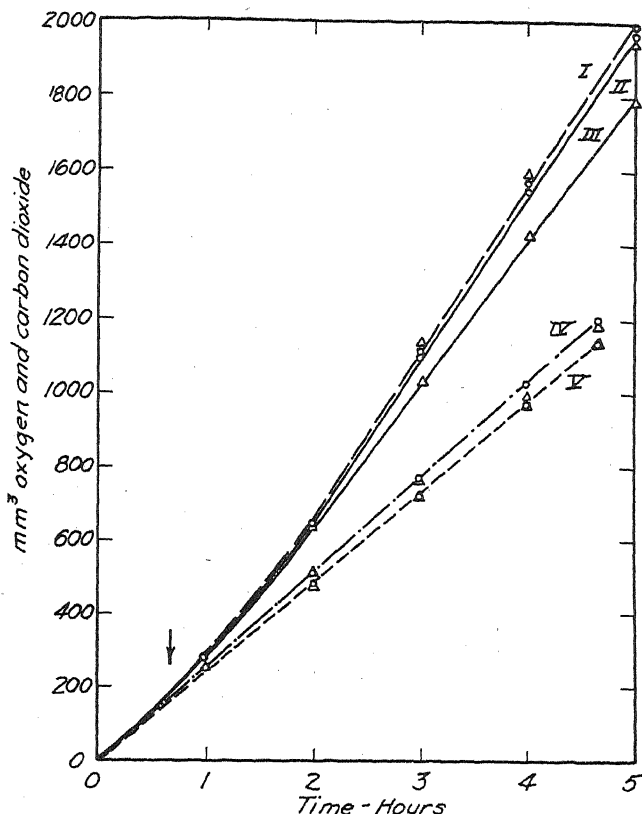


FIG. 1

Influence of Added *p*-Cresol, Xylenol and Tyrosine on the Respiration of Sweet Potato Slices

Thirty slices, washed for 20 hours in running tap water, were used in each of the Warburg flasks. After the slices had been permitted to respire for 40 minutes 2 mg. of each of the monohydric phenols were added to their respective flasks.

Circles indicate O₂ uptake and triangles CO₂ given off.

Curve I represents both the O₂ uptake and CO₂ liberated when xylenol was added. Curve II corresponds to the O₂ uptake and Curve III the CO₂ given off when *p*-cresol was added. Curve IV corresponds to O₂ uptake and CO₂ evolved when *l*-tyrosine was used. Curve V represents the rates of O₂ uptake and CO₂ evolved in the control in which no monohydric phenol was added.

adjacent to the terminal oxidase. It is questionable, however, whether or not *p*-cresol is the natural precursor of the hydrogen carrier adjacent to the tyrosinase in the respiration chain of the sweet potato cells. So far the hydrogen carrier in these cells has not been isolated and identified.

Besides *p*-cresol, being qualified to take part in the respiration of the sweet potato slices, phenol, xynol (4,5-dimethylphenol) and tyrosine also were found, as the data in Fig. 1 show, to play similar roles. The apparent greater effectiveness of the xynol in stimulating the respiration than *p*-cresol, whereas phenol and tyrosine are less so, is possibly due to the difference in their respective affinities for the enzyme (4).

It is known that the rate of oxidation of phenols, by means of tyrosinase *in vitro*, is a function not only of the particular phenol being oxidized, but also of the concentration of the substrate (4). A similar situation seems to prevail when phenols are oxidized by the enzyme *in vivo*. Thus, as the data in Fig. 3 show, there is an optimum concentration of *p*-cresol, between 1 and 3 g. in the reaction volume used, where the rates of the oxidizing and reducing reactions were so balanced that the R.Q. was close to one. This optimum concentration seemed to vary slightly for different sweet potatoes. When concentrations below the optimum were used, then the reducing system appeared to be less active than the oxidizing system and the R.Q. fell below one. With concentrations greater than the optimum, an increase in the amount of carbon dioxide liberated occurred together with an inhibition of rate of oxygen uptake. At present this increase of the amount of carbon dioxide given off, over the amount of oxygen consumed is difficult to explain. Possibly the larger concentrations of *p*-cresol exert some kind of influence on the earlier stages in the carbohydrate metabolism, as for example stimulating aerobic fermentation.

Experimental details. Sweet potatoes in a good firm condition with no signs of decay were used. They were cut into slices of about 1 cm.² and 400 μ thick, and washed in running tap water from 12 to 48 hours before using, similarly to the method used by Baker and Nelson (2) and Boswell and Whiting (1) for the common potato. The temperature of the tap water varied from 10 to 20°C. The dry weight was about 200 mg. for 25 slices. In comparable experiments the slices were always prepared from the same sweet potato.

A Warburg respirometer was used for following the rate and amount of oxygen uptake, and the carbon dioxide liberated was determined according to the direct method of Dixon (5). The temperature of the manometer bath was 25°C. The

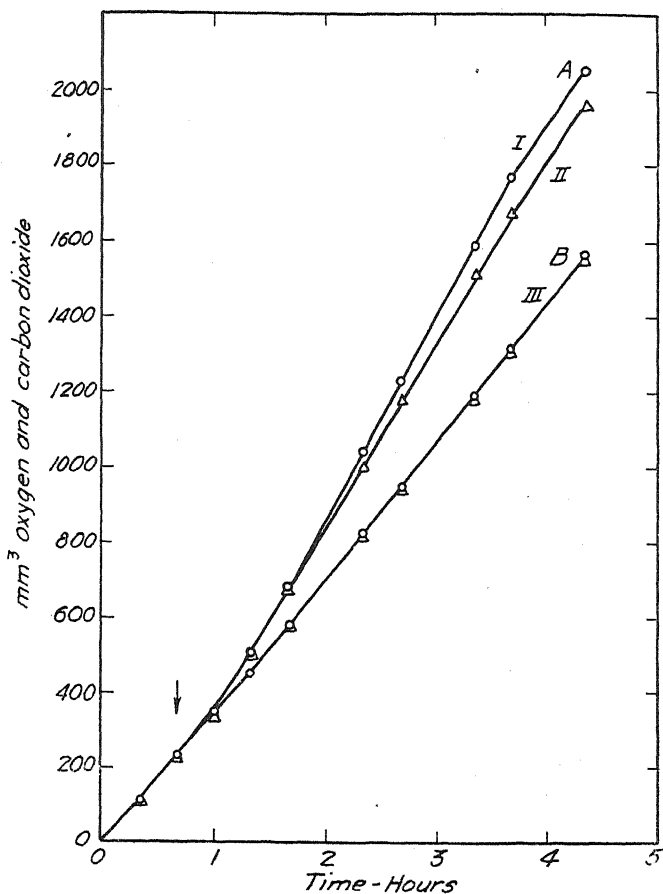


FIG. 2

p-Cresol Acting as a Reservoir for the Hydrogen Carrier, Homocatechol,
Next to the Tyrosinase

Fifty sweet potato slices, washed 20 hours in running tap water, were used.

Circles represent O₂ uptake and triangles CO₂ liberated.

Curve I corresponds to the O₂ uptake and Curve II the CO₂ given off when 1 mg. of *p*-cresol was added, after the slices had been in the respirometer 40 minutes. Curve III represents O₂ uptake and CO₂ evolved in the control. The difference between points A and B corresponds to 488 mm.³ of O₂. Only 310 mm.³ of O₂ were required to oxidize 1 mg. *p*-cresol to its final state of oxidation, hydroxyquinone.

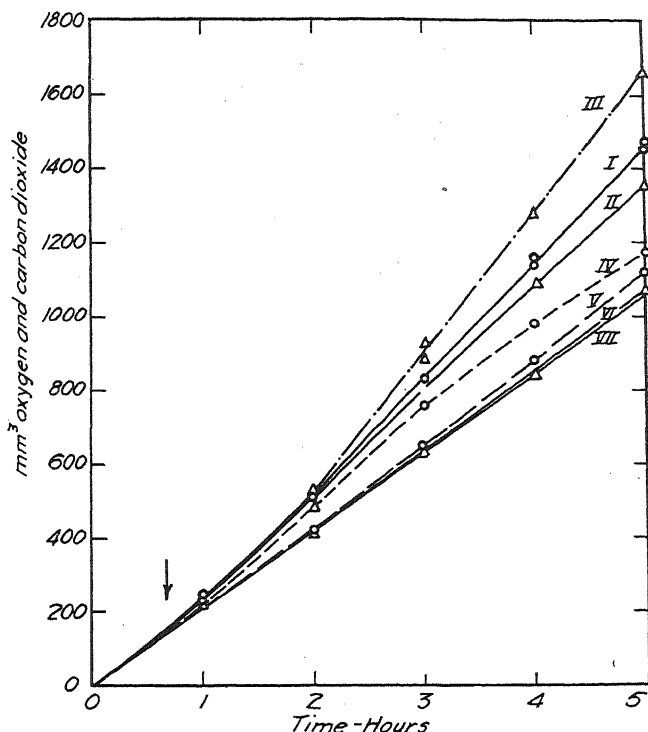


FIG. 3

The Effect of Concentration of *p*-Cresol on Respiring Sweet Potato Slices and the Effect of 3 mg. of Phenol

Circles O_2 uptake and triangles CO_2 liberated.

One and 5 mg. *p*-cresol added after the slices had been permitted to respire in the Warburg flasks for 40 minutes. Twenty-five slices, washed 20 hours in running tap water, were used in each flask.

Curve I represents O_2 uptake when 1 and 3 mg. of *p*-cresol were used. Curve II represents CO_2 given off when 1 mg. *p*-cresol was added. Curve III corresponds to the CO_2 liberated when 3 and 5 mg. of *p*-cresol were used. Curve IV corresponds to O_2 uptake when 5 mg. *p*-cresol had been added. Curve V represents O_2 uptake and Curve VI CO_2 given off when 3 mg. phenol was added. Curve VII represents O_2 uptake and CO_2 liberated in the control in which no monohydric phenol was added.

capacity of the Warburg reaction flasks was about 50 cc. Twenty-five or fifty wet slices were used in the flasks with 5 cc. of 0.013 molar phosphate buffer at pH 5.3 to 5.5. To this mixture were added from the side arm, at the end of the control period, the solutions of the monohydric phenols. Filter paper moistened with 0.2 cc. of 20% potassium hydroxide for absorbing the carbon dioxide, as it was formed, was placed in the center wells, and the total volume including that of the slices was made up to 9.2 cc. with water. The rate of shaking of the Warburg apparatus was 120 complete oscillations per minute and the respiration rate was independent of the rate of shaking.

SUMMARY

1. It has been shown that in the case of sweet potato slices, as well as in the case of slices of the common potato, tyrosinase has the role of a terminal oxidase.

2. The monohydric phenol activity of tyrosinase is important in the respiration of the cells of the sweet potato just as it is in the case of the cells of the common potato tuber. However, all the monohydric phenols tried, *p*-cresol, xylenol, phenol and tyrosine can act as substrates for the tyrosinase in sweet potato cells in contrast to the cells of the common potato where tyrosine but not *p*-cresol acts as the substrate.

3. Monohydric phenols can serve as reservoirs for keeping the respiratory chain in the sweet potato cells supplied with the *o*-dihydric phenols acting as hydrogen carriers adjacent to the terminal oxidase, tyrosinase.

4. The concentration of the monohydric phenols is a factor in the ratio of the rates of the oxidizing and reducing reactions taking place between the preceding part of the respiratory chain and the hydrogen carrier adjacent to the terminal oxidase.

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Studies on the Nicotinic Acid Content of Coffee *

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INTRODUCTION

In the course of routine analysis of army rations, solubilized coffee powder was found to contain enough nicotinic acid to warrant further investigation. Additional microbiological studies were made, and coffee preparations were assayed with chicks and dogs. The pharmacological effects produced in these animals by coffee extract prompted us to study its effect on the rat. This report summarizes our observations.

EXPERIMENTAL

Nicotinic acid assays were first carried out according to the microbiological method of Snell and Wright (1). Table I shows that nicotinic acid is very readily extracted from roasted coffee. Three different brands of roasted coffee were analyzed microbiologically and were found to contain the following amounts of the vitamin: 10.9, 9.5, and 8.0 mg./100 g. A de-cafeinated coffee contained 4.5 mg./100 g.

TABLE I
Extraction of Nicotinic Acid from Roasted Coffee

Procedure	mg. % nicotinic acid
Coarse grind—cold H ₂ O, 10 minutes	7.0
Coarse grind—cold H ₂ O, 3 hours	9.1
Fine grind—cold H ₂ O, 3 hours	10.3
Fine grind—1 N NaOH—autoclaved 30 min., 15 pounds	10.4

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

We are indebted to G. M. Briggs, Jr., and T. D. Luckey for performing the chick experiments, and to Professor K. P. Link for furnishing the crystalline quinic acid and chlorogenic acid used in our studies.

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Coffee beverage purchased at five different restaurants was found to contain the following amounts of nicotinic acid per cup: 0.78, 0.91, 0.95, 0.91, 1.28 mg. Each cup contained approximately 175 cc.

Green coffee was found to be quite different from roasted coffee with respect to nicotinic acid activity (Table II). Water extraction of green coffee yields only one-

TABLE II

The Effect of Autoclaving Coffee with Alkali on its Nicotinic Acid Activity for L. arabinosus

Roasted coffee

Filtrate			Direct			Direct		
		mg./100 g.			mg./100 g.			mg./100 g.
H ₂ O	1 hr.	9.8	2 N NaOH	2 hrs.	14.8	H ₂ O	1 hr.	9.2
2 N NaOH	2 hrs.	10.1	2 N NaOH	4 hrs.	17.6	1 N NaOH	1 hr.	10.2
2 N NaOH	8 hrs.	10.3	2 N NaOH	8 hrs.	23.0	5 N NaOH	1 hr.	17.0
2 N NaOH	12 hrs.	10.0	2 N NaOH	12 hrs.	23.0			

Green coffee

Filtrate		mg./100 g.	Direct		mg./100 g.
H ₂ O	1 hr.	1.04	H ₂ O	1 hr.	1.30
0.1 N NaOH	1 hr.	1.36			
1 N NaOH	1 hr.	2.20	1 N NaOH	1 hr.	6.60
5 N NaOH	1 hr.	2.80	5 N NaOH	1 hr.	9.50

tenth as much nicotinic acid (measured by *L. arabinosus*) as similar extraction of roasted coffee. The extract from green coffee contains material which can be activated for *L. arabinosus* by treatment with moderately strong alkali. The material in green coffee seems too easily activated to be trigonelline (2), and not labile enough to be the derivatives found in wheat bran (3). Rather, it behaves more like compounds of the nikethamide type (4).

Direct treatment of green coffee with strong alkali yields more nicotinic acid than water extraction, but similar treatment of roasted coffee also gives higher values. Completeness of extraction is involved in both cases, and since the water extract of roasted coffee is not affected by alkali treatment, we do not know whether or not activation is involved in treatment of roasted coffee beans with strong alkali.

At any rate, it is evident that the roasting process causes a definite change in the nicotinic acid derivatives in coffee, and our data indicate that compounds in addition to trigonelline may be involved.

Since coffee could contribute a significant portion of the human nicotinic acid requirement if the material measured by *L. arabinosus* were utilized, attempts were made to measure the nicotinic acid in coffee biologically.

Chick Studies

Chicks were placed on the nicotinic acid-low ration of Briggs, *et al.* (5). Nicotinic acid was fed at levels of 0.5, 1.0, 1.5 mg. per 100 g. of ration to obtain a standard curve, and to two other groups, a water extract of roasted coffee was supplemented at two levels calculated to fall within this range. Five chicks were used per group. Both levels of coffee extract produced erratic growth, and the higher level, equivalent to 1 mg. nicotinic acid per 100 g. ration, according to microbiological assay, actually caused slightly poorer growth than was obtained on the basal ration.

In an attempt to eliminate this toxic effect, a purified preparation was made from a water extract of roasted coffee by adsorbing the nicotinic acid on norite (Darco G-60) at pH 3.5 and eluting with a pyridine-methanol mixture. The solvent was removed by vacuum distillation and the preparation was fed at levels equivalent to 0.2 mg. and 0.5 mg. nicotinic acid per 100 g. of ration as determined by microbiological assay. The lower level produced no measurable growth effect, and the higher level caused only one-fifth of the growth response expected in view of the microbiological assay of the preparation. Either the material was only one-fifth as active for the chick as for *L. arabinosus*, or the preparation was toxic enough to prevent full response to the nicotinic acid.

Dog Studies

Nicotinic acid deficiencies were produced in dogs by feeding the following synthetic ration: sucrose 66%, cottonseed oil 11%, acid washed casein 19%, salts 4%. Vitamin supplements were given by pipette at the following levels (mg./kg./day): thiamine 0.1, riboflavin 0.1, pyridoxin 0.06, calcium pantothenate 0.5, and choline 50. Haliver oil was fed at a level of 3 drops/kg./wk. A folic acid concentrate was supplemented at a level equivalent to 0.8 g. solubilized liver powder per day.

It was found to be almost impossible to feed a water extract of coffee successfully because of its strong emetic action. Therefore, in most of the work with dogs, a norite eluate prepared as described above was used. This preparation was fed by stomach tube and rarely produced vomiting.

To three nicotinic acid deficient dogs, the following supplements were given respectively: (a) norite eluate equivalent to 180 g. roasted

coffee, (b) a similar preparation equivalent to 90 g. roasted coffee, (c) water extract of 140 g. roasted coffee. In no case was there any response in the next 24 to 48 hours. Neither was there any response to from 25 to 50 mg. of nicotinic acid in the following 24 to 48 hours. After these two supplements had failed to cure the animals, 50 mg. *p*-aminobenzoic acid, 50 mg. inositol, and 30 μ g. biotin concentrate was given to the first dog. A rapid and complete recovery was obtained. The second dog developed a severe "weepy eye" condition characterized by much thick exudate, marked conjunctival hyperemia and corneal opacity. Administration of 60 μ g. of crystalline biotin cured the eye condition and produced a growth response equal to what was expected from the nicotinic acid that had been fed. The third dog also made a complete weight recovery on administration of 50 μ g. crystalline biotin.

An assay in which *p*-aminobenzoic acid, inositol, and biotin were supplied to a blacktongue dog and "nicotinic acid" in coffee eluate was fed together with crystalline nicotinic acid at a ratio of 2:5 indicated that the material in coffee was equally active for the dog and for *L. arabinosus*.

Finally a more highly purified preparation was made from roasted coffee. From 160 g. of dried coffee extract, a norite eluate was prepared using 20% NH_4OH instead of the pyridine-methanol mixture for elution. The eluate was concentrated to 150 cc., and absolute alcohol was added to bring the ethanol concentration to 90%. The material was cooled to 5°C., filtered, and the filtrate was taken to dryness. This final preparation contained 78% of the activity for *L. arabinosus* found in the starting material.

A blacktongue dog was obtained with a basal ration containing biotin, *p*-aminobenzoic acid, and inositol. An amount of the new preparation was fed which contained 28.2 mg. of nicotinic acid according to microbiological assay. The response of the dog indicated the presence of 27.9 mg. of nicotinic acid.

It is of interest to note that, while "folic acid" has been found to enhance the response of blacktongue dogs to crystalline nicotinic acid (6), biotin, *p*-aminobenzoic acid, and inositol have been found to be without effect under ordinary conditions.

Dogs placed on the basal ration plus nicotinic acid with added roasted coffee or coffee extract have displayed a variety of symptoms. Diuresis and catharsis have been observed in varying degrees in all the dogs. A seven week old dog receiving coffee extract in the ration

equivalent to 5% roasted coffee lost weight and died within four days. An older dog did not show any effects when placed on the above ration for several weeks and 5% finely ground roasted coffee was substituted for the extract. In 8 days a flaccid paralysis developed in all quarters although the dog could move his head freely and was mentally alert. After 24 hours, 200 mg. of inositol was fed, and within the next day the dog became normal. The level of coffee was raised

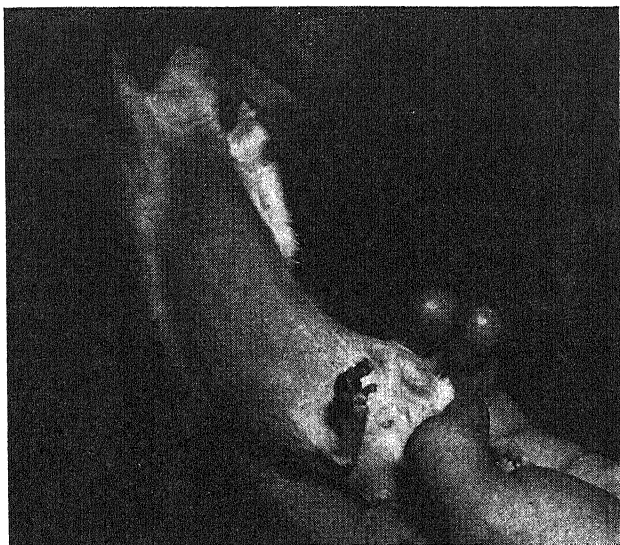


FIG. 1

Alopecia Caused by the Addition of 10% Finely Ground Roasted Coffee to a Synthetic Ration

to 7.5% and within one week the dog's hair began turning from its reddish brown color to gray. The level of coffee was raised to 10% and the hair grayed more rapidly, the animal lost weight, and the eye condition described above developed. Fifty μ g. of crystalline biotin was fed, and the eye symptoms disappeared in three days. The coffee was taken out of the ration, and the animal increased its food consumption and soon appeared quite normal except for the coat.

Another dog was placed on the basal ration plus 5% roasted coffee for four weeks with no apparent effects. The level was raised to 10%, and in two days the same type of paralysis described above developed.

Feeding of 100 mg. inositol produced recovery within 24 hours. A week later the dog had convulsions, and the level of coffee was reduced to 5 per cent. A month later convulsions were again observed, growth was very poor, and the animal finally died of a respiratory infection.

In our experience, dogs placed on the above basal ration plus nicotinic acid and without coffee do not show any of the conditions described above.

Rat Studies

In view of the results obtained with chicks and dogs, it was deemed advisable to extend our studies to the rat. In experiments where growth rates are reported, 21-day-old, male, albino Sprague-Dawley rats were used, three rats per group. Other studies were made with stock rats and it was found that all of our results could be observed in rats of either sex. A total of 225 rats were used.

The basal ration consisted of:

Sucrose	76 per cent
Labco casein	18 " "
Salts IV	4 " "
Corn oil	2 " "
Choline	100 mg./100 g.
Nicotinic acid	0.250 " / " "
Ca Pantothenate	2.0 " / " "
Thiamine	0.200 " / " "
Pyridoxin	0.250 " / " "
Riboflavin	0.300 " / " "
2 drops of haliver oil were fed weekly to each rat.	

Preliminary work brought out the following facts: If finely ground roasted coffee replaced 50% of the above ration, the rats developed a severe edematous condition in the extremities, lost weight and died within a few days. At a level of 20% coffee, the animals developed a similar condition in lesser degree and died within two weeks. At a 10% level, animals survived but growth was very poor. In some cases alopecia occurred about the belly, neck, fore-paws, and hind quarters, ranging in extent from small patches to complete loss of hair in the areas mentioned. A 5% level of coffee also produced some cases of alopecia with fair growth. The alopecia is usually of a "migrating" nature, with new bare spots developing as old ones are filled in. We

severely found variation among roasted coffees in their ability to produce ing's condition. If alopecia develops at a 5% level, it tends to cure spontaneously after about two weeks, but raising the level of coffee to 10% again produces loss of hair. Loss of hair has been observed in about one-fourth of the rats receiving the above levels of coffee and has never been observed on the basal ration without coffee. Addition of inositol to the ration seems to be of some help but it does not completely cure nor prevent the alopecia. Neither does 3% 1:20 liver powder afford complete protection. In some cases, the loss of hair can be observed within two days after rats are placed on rations containing coffee.

We have observed growth depression and edema on the addition of similar levels of coffee to our stock ration but have not studied enough animals to know whether or not alopecia might be produced on a ration of this type.

One series of rats contained a group which received 5% coffee plus 0.5% inositol from the beginning of the experiment. Table III shows

TABLE III
Average Weekly Gain of Rats During First Four Weeks on Experiment

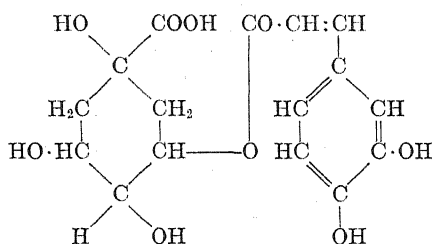
	Basal ration g.	+5% Coffee g.	+5% Coffee + 0.5% inositol g.
1st week	24	4	20
2nd week	27	11	25
3rd week	29	21	20
4th week	32	22	22

that inclusion of inositol improved growth markedly during the first two weeks but thereafter there was no effect. In this series, one rat in the 5% coffee group and two rats in the group receiving both coffee and inositol developed mild alopecia. Other groups in this series demonstrated that a water extract of roasted coffee produces alopecia and retards growth, though it does not appear to be as potent as whole coffee. Green coffee and decaffeinated coffee retarded growth to a lesser extent than did roasted coffee, but since we have found variation among roasted coffees, it is impossible to make quantitative comparisons from our studies.

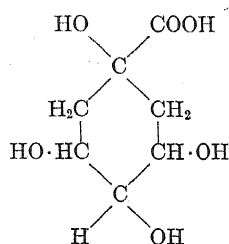
Other experiments have shown that when coffee is fed at a 10% level, neither inositol nor 5% whole liver substance, nor a combination of the two is able to counteract the growth depression. Inositol was

fed at levels ranging from 0.05% to 4.0%. These amounts added to the basal ration without coffee had no effect on growth. It has been found that up to 15% whole liver substance, with or without added inositol, will not cure the alopecia.

The occurrence of alopecia suggested that there might be some interference with inositol utilization. The resemblance of quinic acid and chlorogenic acid to the inositol molecule suggested that there might be a competitive effect. Green coffee contains about 10% chlorogenic acid and on roasting, about half of it is broken down to quinic acid and caffeic acid (7).



Chlorogenic acid



Quinic acid

Crystalline quinic acid was fed at levels ranging up to 0.5% of the diet and chlorogenic acid at levels up to 1.0% of the diet for two weeks with no growth depression or alopecia production. However, longer periods of feeding with the addition of other constituents of coffee might produce positive results.

Crystalline caffeine was fed to several series of rats and both alopecia and growth depression were observed, but these symptoms were less

TABLE IV
Comparative Growth Rates of Rats Receiving Coffee and Caffeine Supplements
3 wk. growth period

Ration	Gained per week g.
Synthetic ration	31
" " + 5% roasted coffee	23
" " + 10% roasted coffee	12
" " + 0.05% caffeine	27
" " + 0.1% caffeine	25
" " + 0.25% caffeine	17
" " + 5% decaffeinated coffee	22
" " + 10% decaffeinated coffee	16

severe than those produced by an equivalent amount of coffee, assuming the latter to contain 1-2% caffeine. 0.5% caffeine produced the same type of edema and early death that high levels of coffee did. Table IV gives typical growth data. It should be noted that decaffeinated coffee produced growth depression, but we have never observed loss of hair in rats receiving decaffeinated coffee.

DISCUSSION

Our results with blacktongue dogs indicate that coffee drinking may produce a number of effects in humans. In individuals on poor diets, ingestion of coffee may help produce vitamin deficiencies just as a biotin deficiency is apparently precipitated in the blacktongue dog. However, if this effect is obviated, coffee may actually be able to prevent nicotinic acid deficiency in humans, and it seems reasonable to assume that individuals in a fairly good nutritional state could utilize the several mg. of nicotinic acid that would be furnished by drinking several cups of coffee per day.

In discussing nutrition in Brazil, De Castro (8) states, "There is no pellagra zone in the country, this disease being a clinical rarity." Extensive coffee consumption may account for this fact, since the average Brazilian diet seems quite low in nicotinic acid and contains the classical "pellagra-producing" maize.

The importance of avoiding coffee when individuals are on balance studies has been recognized because of the interference of trigonelline, but further complications would arise because of the existence of compounds with nicotinic acid activity. This would be especially important in the correlation of the incidence of pellagra with the diet, and it may account for some of the discrepancies which have shown up in work of this nature in the past.

It is difficult to theorize as to how important the symptoms we have observed in the chick, dog, and rat may be in the case of the human. Perhaps the most significant fact we have observed is that the amounts of coffee extract which produce growth depression in experimental animals approach amounts consumed by some humans. Beyond this, the toxic effects vary with the species and the possible effect on the human is purely a matter of conjecture.

It is true that most of our work has been done with synthetic rations, but some of the symptoms have been produced on a stock

ration and large quantities of dried liver have not been able to prevent the toxic manifestations in rats.

The results we have obtained might be due to effects on the intestinal flora, interference with absorption of nutrients, direct effects in the tissues, or a combination of these factors. Thus it seems apparent that much work is necessary if the exact mechanisms are to become known.

SUMMARY

1. Roasted coffee was found to contain about 10 mg. of nicotinic acid per 100 g. Restaurant coffee contained about 1 mg. per 175 cc. cup.

2. In contrast to roasted coffee, a water extract of green coffee contains considerable amounts of nicotinic acid derivative(s) which are activated for *L. arabinosus* by treatment with moderately strong alkali.

3. An attempt was made to measure the nicotinic acid activity of coffee for the chick, but the amounts of coffee extract fed seemed to be toxic.

4. When coffee extract or a norite eluate of coffee extract was fed to blacktongue dogs on a synthetic nicotinic acid low diet, a biotin deficiency was apparently precipitated. The condition was characterized by lack of response to nicotinic acid administration and the development of an eye condition which responded to treatment with crystalline biotin.

5. By inclusion of biotin in the basal diet, a satisfactory dog assay was obtained, and the results indicate that the material measured by *L. arabinosus* is equally active for the dog.

6. Dogs receiving 5-10% finely ground roasted coffee in the diet developed a number of symptoms including the eye condition mentioned above and a paralysis involving all quarters which responded to inositol administration.

7. Addition of 5-10% roasted coffee to the diet of rats produced poor growth with loss of hair in some cases. Caffeine also produced alopecia, but was not as potent as equivalent amounts of coffee in depressing growth. Decaffeinated coffee retarded growth but did not produce alopecia.

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The Colorimetric Determination of Pentoses and Pentosans

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INTRODUCTION

Certain methods in common use for the quantitative determination of pentosans in plant material depend upon conversion of the polysaccharide to furfural in the presence of strong acid, and gravimetric (1) or colorimetric estimation of the aldehyde after it has been distilled out or removed with an immiscible solvent (2). Since these operations are not well suited to routine analyses, alternative procedures have been sought when it is necessary to handle many samples. Thus it is feasible in some instances to hydrolyze the polymeric sugar to free pentose, ferment out interfering hexoses with yeast, and determine the residual reducing substances as pentose (3). The present paper describes a method embodying certain features of both these general procedures.

Briefly this method consists in removing soluble sugars from the dry material by alcohol extraction, hydrolyzing the residue to solubilize pentosans, fermenting out interfering substances when necessary, and assaying the resulting solution by a simple colorimetric test. Although several steps are involved in this procedure, they are of such a nature as to be applicable on a routine basis.

EXPERIMENTAL

Color development. The colorimetric determination is based upon one of the numerous color reactions of the 5-carbon sugars, namely the interaction of pentose with orcinol in the presence of ferric chloride and concentrated hydrochloric acid (Bial's

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reaction). The operations involved in using these reagents are relatively simple, and the green color produced is quite satisfactory for colorimetry. A careful study has been made of the conditions affecting the stability of the reagent and the development of color, with considerable improvement over the procedure of Mejbaum (4).

Reagents. Orcinol reagent. Two g. of orcinol are dissolved in 50 ml. of 1.5% ferric chloride (hexahydrate) solution. Sufficient 30% hydrochloric acid (concentrated acid diluted with 1/5 vol. of water) is added to make 1 liter. This reagent, which is stable for at least 4 days, should be stored in a dark bottle and kept out of contact with cork. It is conveniently dispensed from a 15 ml. automatic pipette.

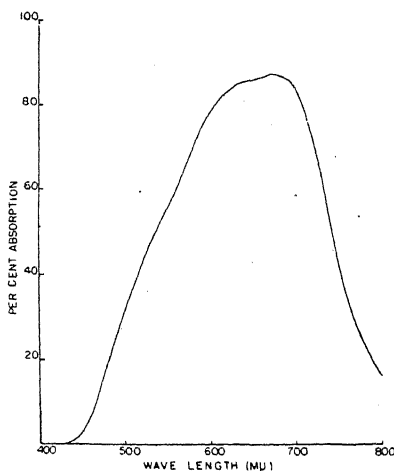


FIG. 1

Absorption Curve of the Colored Compound Formed by the Interaction of Xylose with Orcinol in the Presence of Hydrochloric Acid and Ferric Chloride

Pentose standard. One hundred mg. of xylose are dissolved in 500 ml. of water. This stock solution is stored in the refrigerator and an aliquot diluted 1 : 10 for the working standard.

Procedure. In separate test tubes are placed 5 ml. of the solution to be analyzed (preferably diluted to contain less than 3 mg. % pentose), 5 ml. of the 2 mg. % xylose standard, and 5 ml. of water. Fifteen ml. of the orcinol reagent are added to each tube, the contents mixed by inversion, and the tubes placed in a boiling water bath for 20 minutes. After cooling in a tap water bath, the optical densities of the contents are determined in a photoelectric colorimeter equipped with a red (660 mμ) filter, the blank solution serving as the reference. Due to the high stability of the colored compound, standing time is not critical. The Klett-Summerson colorimeter (research model) was used in these studies, and Beer's law was observed to obtain up to a concentration of 8 mg. % xylose when the 2.5 mm. Klett solution-cell was employed. However, tubes supplied with the instrument are more convenient to handle than

the cell and greatly enhance the sensitivity of the measurement by providing a greater thickness of solution, but the range over which Beer's law appears to hold is correspondingly reduced. (Because of wide variations among instruments it is necessary to determine the effective limits of concentration at a given solution thickness for each colorimeter.) From the readings of the standard and unknown, the concentration of the latter is calculated and pentosan values expressed as xylose.

The factors involved in the choice of light filter were dictated partly by a consideration of the absorption characteristics of the green color produced in this reaction (Fig. 1). The data for this curve were obtained with a Coleman spectrometer, measurements being made at

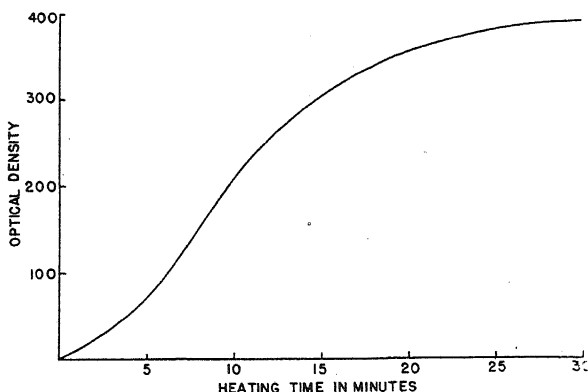


FIG. 2

Curve Showing the Relation between Optical Density and Heating Time for the Reaction between Xylose and Orcinol in the Presence of Hydrochloric Acid and Ferric Chloride

25 $m\mu$ intervals over the range 350–800 $m\mu$. A 660 $m\mu$ filter with an approximate spectral range of 640–700 $m\mu$ effectively transmits light in the region of maximum absorption for the colored compound being measured. This filter serves the additional important function of eliminating even the very small optical interference introduced by the presence of plant pigments in the extract taken for analysis.

In Fig. 2 are plotted data showing the effect of heating time on the resulting optical density. A 20 minute heating time was arbitrarily chosen, this period being outside the region of highest sensitivity with respect to the time factor; it is clear, however, that both standard and unknowns must receive identical treatment in this respect. Shorter

heating times may be used with a resultant increase in the range of concentrations over which Beer's law holds, but with diminution in the sensitivity of the test.

APPLICATION TO THE DETERMINATION OF PENTOSANS IN PLANT MATERIAL

The application of the foregoing reaction to the determination of pentosans in a given type of plant material must be preceded by a consideration of possible interfering substances. In this connection the most important compounds likely to occur in acid extracts of plant material (that has been previously extracted with alcohol) are glucose, fructose, galactose, uronic acids, and the pentoses to be determined, these materials arising from polysaccharides and appearing as hydrolysis products. The approximate chromogenic value of these derivatives on a weight basis with pentose (xylose) assigned the value of 100% is, glucose 1%, fructose 4%, galactose 5%, and glucuronic acid 44%. When the sugars occur in significant amounts in the acid extract they may be readily fermented out, but the quantitative removal of uronic acids from pentoses has not proven feasible; the method therefore does not distinguish between these two classes of compounds. However the relative concentrations of these substances are usually so distributed as to minimize or render insignificant the errors introduced by uronic acids. (The latter may be determined in the presence of pentoses by Tollens' test as modified by Diechmann (5), by Ratish and Bullowa (6), or by others.)

Procedure. One-gram samples of the dry, ground plant material are continuously extracted with 80% ethanol for 6 hours, and the residues dried by placing the thimbles in an oven at 70–80° for a short period. The dry material is then quantitatively transferred to 200 ml. volumetric flasks, about 25 ml. of 1 *N* H₂SO₄ added (3), and the flasks placed in a boiling water bath for 10–15 minutes to "wet" the tissue. An additional 75 ml. of acid is then added and the hydrolysis allowed to proceed at 100° for a total of 3 hours, or for such time as is necessary to obtain maximum pentosan values; this time will vary somewhat with the nature of the material being analyzed and should be experimentally determined on a typical sample. (Comparative results may be obtained by arbitrarily defining the hydrolysis conditions even though maximum yields are not obtained.) Following hydrolysis, the contents of the flasks are cooled, made to volume, and a portion filtered. After appropriate dilution, this filtrate is analyzed as described under color development. No significant error is introduced by omission of further washing of the residue or clarification of the filtrate.

Removal of hexose sugars by fermentation is rarely required because the distribution of pentosan in relation to interfering polysaccharides tends to eliminate errors introduced by the latter. The necessity for performing this step is determined as follows: a typical sample of material is extracted as above and 20 ml. of the filtrate neutralized, using phenolphthalein as indicator. A yeast preparation is made by thoroughly washing baker's yeast at the centrifuge and suspending the packed cells in an equal volume of phosphate buffer, pH 6.8. Four ml. of the yeast suspension are added to the neutral filtrate and the mixture allowed to ferment for 2-3 hours, preferably in an incubator at 38°. After diluting to 50 ml., a portion is centrifuged and the supernatant analyzed for its pentose content. The value so obtained is compared with a non-fermented control, and considering the method reproducible to within $\pm 5\%$ of the amount determined, significant differences taken to indicate that routine fermentation is required. Material requiring this treatment has not been encountered by the writers.

As an index of the validity of the method as applied to plant tissues a recovery experiment was carried out in which plant extracts were supplemented with known amounts of xylose. In Table I are shown

TABLE I
Recovery of Pentose Added to Plant Extract

Pentose present in plant extract (guayule) <i>mg. per 100 ml.</i>	Pentose added <i>mg.</i>	Pentose recovered <i>mg.</i>	Per cent recovery of added pentose
0.66	0.50	0.49	98
"	1.00	0.99	99
"	1.50	1.53	102
"	2.00	2.02	101
"	2.50	2.45	98

typical data, with recoveries well within the limits of reproducibility of the test. Optical density measurements were made using tubes supplied with the Klett-Summerson instrument rather than the rectangular cell in order to obtain high sensitivity.

This method has been successfully applied to the analysis of the leaves, stems, roots, flowers, and seeds of guayule, and to vegetative tissues of apple and of ryegrass. Data on the latter material obtained by furfural distillation and by determination of reducing power after fermentation agreed well with the results of the colorimetric method.* The colorimetric procedure can also be used for the analysis of pentose derivatives such as partially purified nucleotide preparations, where

* Ryegrass samples and data kindly furnished by Dr. J. T. Sullivan, U. S. Dept. of Agriculture.

its high sensitivity permits evaluation of very small samples. The method is not applicable in the presence of nitrates.

SUMMARY

A sensitive colorimetric determination of pentoses based upon Bial's reaction is described, and the application of this test to the quantitative estimation of pentosans in plant material is outlined. A method is given for the elimination of errors introduced by certain common plant constituents which might be expected to occur in the extracts taken for analysis.

The procedure is reproducible to within $\pm 5\%$ of the amount of pentosan determined.

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Stereochemical Configuration and Provitamin A Activity III.* All-*trans*- α -carotene and neo- α -carotene U

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INTRODUCTION

The first data concerning the provitamin A activity of isomerized α -carotene were published by Gillam, El Ridi and Kon (5). From natural α -carotene they obtained a "neocarotene" solution which upon crystallization yielded "neo- α -carotene." Neocarotene was found qualitatively to be active (4 rats) while neo- α -carotene showed about 70% of the potency of β -carotene (8 rats). None of these pigments has been identified with any pure α -carotene stereoisomer described recently (8).

In the present study we have determined the ratio of the provitamin A activity of all-*trans*- α -carotene to that of a well-crystallized, stable isomer, neo- α -carotene U. The latter is very probably 9-mono-*cis*- α -carotene (or, possibly, 3-mono-*cis*- α -carotene). It was found earlier (2) that a single *trans* \rightarrow *cis* rotation (all-*trans*- β -carotene \rightarrow neo- β -carotene U) reduces the A-activity in the rat by about 60 per cent. On the other hand, pro- γ -carotene, a naturally occurring stereoisomer of γ -carotene, possesses a greater biological activity than the all-*trans* compound (from *Mimulus*) (3).

The relative effectiveness of α - and β -carotene has not yet been definitely established. Kuhn, Brockmann, Scheunert and Schiebllich (7) have found the minimal effective daily dose ("Grenzdosiz") for the rat to be 5 μ g. of α -carotene and 2.5 μ g. of β -carotene. However, Euler, Karrer and Zubrys (4) have observed a different ratio and a much higher activity for both carotenes. When they fed 0.75 μ g. of

* For Parts I and II, see References 2 and 3.

α -carotene daily, the average daily increase in weight of the rats was 0.48 g., and in parallel assays with β -carotene, 0.72 g. Since it seemed possible that these discrepancies may have been due in part to the differences in the amount of α -tocopherol in the diet (6), we are also reporting on some new experiments in which the ratio of A-activity of α - and β -carotene has been re-determined.

EXPERIMENTAL

Neo- α -carotene U was prepared as described earlier (8). It was dissolved in the Wesson oil which contained α -tocopherol. The extinction of the solution was still unchanged after having been kept in darkness at 5° for four days, and then at 25° for two hours. A sample examined spectrophotometrically after the conclusion of the bioassay showed no marked alteration. The procedures for the assay were described elsewhere (2, 3). Rats from our stock colony were used. After showing signs of vita-

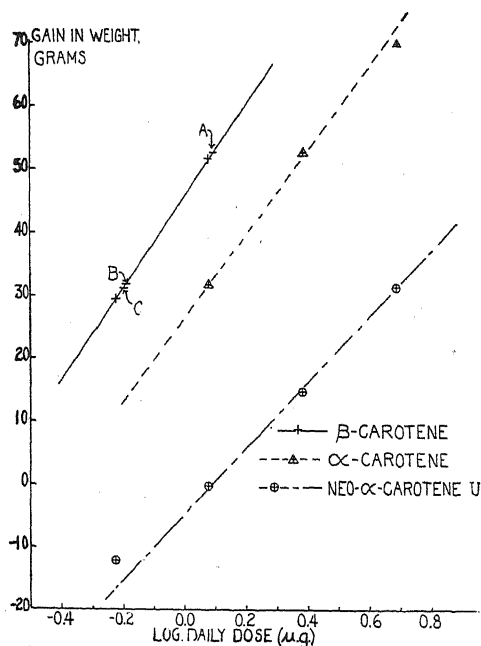


FIG. 1

Relationship of Gain in Weight to log. of Daily Dosage of β -Carotene, α -Carotene, and Neo- α -carotene U

Points A and B represent the projection on the β -carotene curve of the average gain (in grams) for the groups receiving 2.4 μ g. and 1.2 μ g. of α -carotene respectively. Point C represents a similar projection referring to the group which received 4.8 μ g. of neo- α -carotene U.

TABLE I

Summary Table of Bioassay Experiments on Male and Female Rats Receiving β -Carotene, α -Carotene, Neo- α -carotene U, or Cottonseed Oil Only

(The average results on males and females are weighted equally. Where animals died during the course of the experiments, the number of animals still alive which are included in the average is given in parentheses. The average age at the start of the depletion period was 22 days.)

Supplement	Dose per day $\mu g.$	Number of rats		Depletion period			Assay period										Average final weight
				Average weight at start	Average dura- tion days	Average final weight	Average increase in body weight up to the following days					Average increase in body weight up to the following days					
							Male	Female	5th	10th	15th	20th	25th	28th	5th	10th	
β -Carotene	0.6	8	8	41.8	21	84.0	3.4	12.8	20.3	27.2	29.5	29.5	29.5	29.5	29.5	113.4	
	1.2	8	7	43.0	19	86.8	4.4	17.1	27.7	38.6	46.8	51.6	51.6	51.6	51.6	138.4	
	1.2	8	7	41.6	21	87.0	0.6	10.6	18.0	24.6	31.0	31.8	31.8	31.8	31.8	118.8	
α -Carotene	2.4	8	8	42.3	20	85.9	8.4	22.3	34.3	45.1	51.1	52.6	52.6	52.6	52.6	138.5	
	4.8	8	7	42.0	19	83.6	12.1	28.8	44.0	58.4	68.4	69.6	69.6	69.6	69.6	153.0	
	0.6	8	7	42.4	23	93.0	-4.5	-0.2	-2.2	-4.8	-11.0	-12.2	-12.2	-12.2	-12.2	85.2	
Neo- α -carotene U	1.2	8	7	41.2	20	86.7	-0.4	2.3	3.1	-0.2	0.4	-0.3	-0.3	-0.3	-0.3	86.6	
	2.4	8	7	42.6	20	87.5	1.1	8.5	12.2	13.7	16.6	14.6	14.6	14.6	14.6	102.4	
	4.8	7	8	41.8	20	83.8	2.2	10.0	18.2	24.7	28.4	31.2	31.2	31.2	31.2	117.2	
Negative controls	0.0	15	12	42.3	20	85.7	-0.5	-0.2	0.7	-0.1	-6.5	-8.2	-8.2	-8.2	-8.2	78.0	
							(26)	(25)	(22)	(20)	(13)	(14)	(14)	(14)	(14)		

min A deficiency they were assigned to one of ten groups. These each received daily, in addition to the A-free diet, one of the following supplements dissolved in 0.1 ml. of Wesson oil containing 0.5 μ g. of α -tocopherol: 0.6 or 1.2 μ g. of β -carotene; 1.2, 2.4 or 4.8 μ g. of α -carotene; 0.6, 1.2, 2.4 or 4.8 μ g. of neo- α -carotene U; or oil-tocopherol alone (negative control group). A total of 164 rats from 27 litters were used.

After 28 days the gains in weight in the groups which received β -carotene were quite similar to those which had been obtained earlier (2). The average total increases in the new 0.6 μ g. series were 29.5 g. compared with 25.0 g., while the corresponding means in the 1.2 μ g. series were 51.6 g. and 47.9 g. respectively. Concerning the results obtained with α -carotene and neo- α -carotene U we refer to Table I. In the negative control group (which consisted of one rat from each litter) over two-thirds of the animals had begun to lose weight by the tenth day of the assay period and over half of them had died before the twenty-eighth day.

The dosage/gain-in-weight curves are given in Fig. 1. The three points for α -carotene as well as the four points for neo- α -carotene U fall on straight lines which are reasonably parallel to the line passing through the two reference values for β -carotene. This supports the reliability of the data. The potency of α -carotene, calculated by the method of Coward (1), was found to be 53% of β -carotene; *i.e.* 1.9 μ g. (average of 1.86 and 1.93 μ g.) is biologically equivalent to 1.0 μ g. of β -carotene. The A-activity of neo- α -carotene U (calculated on the group receiving 4.8 μ g. daily) was found to be one-fourth of that of all-*trans*- α -carotene, and, consequently, only one-eighth of that of all-*trans*- β -carotene.

DISCUSSION

On the basis of the data obtained, the relative A-activities of six isomeric carotenes are given in Table II. This table shows that the ratio, β -carotene activity/ α -carotene activity, is very nearly 2:1 al-

TABLE II
Relative Provitamin A Activities of Some Carotenes in the Rat

All- <i>trans</i> - β -carotene	100
Neo- β -carotene U	38
All- <i>trans</i> - α -carotene	53
Neo- α -carotene U	13
All- <i>trans</i> - γ -carotene	28
Pro- γ -carotene	44

though the absolute daily doses now given (in the presence of α -tocopherol) were about one-eighth of those administered by Kuhn *et al.* (7). Considering the very different methods and especially the absence of data on the weight increase of rats which received several β -carotene levels in the investigation of Euler, Karrer and Zubrys (4), no direct comparison with their results seems to be possible.

Table II shows that one peripheral *trans* \rightarrow *cis* rotation decreases the vitamin A activity of all-*trans*- β -carotene by 62% and that of all-*trans*- α -carotene by 75% which figures are very similar, considering the margin of experimental error.

SUMMARY

The following relative provitamin A potencies were found in the rat. All-*trans*- β -carotene:neo- β -carotene U:all-*trans*- α -carotene:neo- α -carotene U = 100:38:53:13.

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Fermentation of Wood Hydrolyzates by Yeast and *Fusaria*

In recent investigations carried out in this laboratory, new applications of members of the genus *Fusarium* were established. Certain *Fusaria* compare favorably with brewer's yeast in food value (1), and are also able to increase the alcohol yield of sulfuric acid hydrolyzed wheat mash by 6–12% when the orthodox yeast fermentation of the hexoses present is followed by a fusarial pentose fermentation (2).

The purpose of the present work was the determination of the amount of ethanol obtainable from carbohydrates (hexoses as well as pentoses) present in wood hydrolyzates as their butyl alcohol fermentation had been previously (3) studied.

The material investigated in these experiments was a Douglas-fir hydrolyzate prepared with dilute sulfuric acid and obtained through the courtesy of Dr. E. E. Harris of the Forest Products Laboratory, Madison, Wis. When analyzed at monthly intervals, this hydrolyzate gave the following increasing values for total reducing sugars: 5.84%, 6.11%, and 6.48%. The hydrolyzates were prepared for yeast fermentation by preliminary filtration followed by neutralization to pH 4.5 by sodium or calcium hydroxide, a second filtration, dilutions up to 1 : 5, and sterilizations under pressure.

To one liter of the pretreated hydrolyzate the following salts were added: 1.00 g. KNO_3 , 1.5 g. KH_2PO_4 , 0.75 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The fer-

*Fermentation of Wood Hydrolyzate Neutralized by $\text{Ca}(\text{OH})_2$ by Yeast and *Fusaria* at 31° and 28° C.*

Dilution	Ethanol obtained by yeast in g./100 ml. undiluted hydrolyzate	Ethanol obtained by FIB in mg./100 ml. undiluted hydrolyzate
1 : 5	2.10	26.66
2 : 5	2.10	15.60
3 : 5	1.99	9.77
4 : 5	1.83	6.66
undiluted	1.68	6.77

mentations were carried out with 2 g. of compressed National Grain yeast per 100 cc., at 31° C., and the yields of ethanol obtained after

a period of 24 hours ranged from 53.8% to 67.3%, depending upon dilution.

The yeast-fermented mashings were then filtered and concentrated to small volume *in vacuo* to remove the last traces of alcohol. These concentrates were diluted to their previous volumes and treated with 1-2 g. of Norite per 100 cc. as preliminary experiments indicated the presence of growth inhibiting substances which could be easily adsorbed as in sulfite waste liquors (4). The inoculations with *Fusarium lini* Bolley (FIB) were then carried out as described in earlier communications from this laboratory. In the course of 16 days a moderate growth of the organism was observed at 28° C. The fusarial mats were filtered off and the alcohol determined by a dichromate oxidation method (5). From 6 to 26 mg. of alcohol were obtained from 100 ml. of solution, equivalent to 2.2-4.7% of the alcohol obtainable from the residual carbohydrate content (calculated as pentose) of these hydrolyzates.

These preliminary results would indicate that the "pentose"-alcohol of this wood hydrolyzate amounts to about 1% of the alcohol derived from yeast fermentation.

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Contribution No. 39 from the
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Renin and Kidney Cathepsins

During the course of experiments to separate from each other the kidney cathepsins I, II, III, IV, results were obtained which showed that renin was not identical with any of these four proteolytic enzymes.

Plentl and Page (1) have recently published data which point in the same direction, and it is the purpose of this note to add further and more direct proof to their results.

The synthesis of all substrates used and the incubation experiments were carried out as described by Bergmann and coworkers (2). The hydrolysis of the substrates by cathepsin II and III was followed with the micro-titration procedure of Grassmann and Heyde (3). The activities of cathepsin I and IV were followed by the titrimetric ninhydrin method (4).

Extracts were prepared from dried pig kidney powder as described in a previous paper (5). The first step in this procedure (extraction of dry powder with 2% NaCl solution, followed by centrifugation) yielded clear solutions, in which the presence of all 4 cathepsins was easily detectable. While the activities of cathepsin I and cathepsin IV in such extracts were of the same order as those observed by Plentl and Page (1), we found only about $\frac{1}{10}$ of the amounts of cathepsin III mentioned by these authors. Low cathepsin III activity in kidney extracts had previously been observed by Fruton, Irving, and Bergmann (2).

Further purification according to our method (5) gave the following results.

Cathepsin I: Not present in fraction A and fraction C.

Cathepsin II: Over 90% are lost by going from fraction A to fraction C. If the activity of cathepsin II is expressed by the (first order)

constant $K = \frac{1}{t} \log \frac{a}{a-x}$ and if $R.U.$ = number of renin units (6)

per ml. test solution, the values obtained for $\frac{K \times 10^4}{R.U.}$ were (for fraction A) 2.7 and (for fraction C) 0.21.

Cathepsin III: Small amounts were still present in fractions A and C.

Tests done with the concentrated commercial renin preparation No. 1000 (S.M.A. Corporation) showed, however, that this product did not contain cathepsin III. Renin No. 1000 did show cathepsin I, II, and IV activities.

Cathepsin IV: Not present in fraction C.

It can be concluded from our findings, that renin is not identical with cathepsin I, III, and IV. The ten-fold change in the ratio cathepsin II : renin activity observed while going from fraction A to C indicates that renin is also not identical with cathepsin II, unless one assumes cathepsin II to be a mixture of proteolytic enzymes (with renin being one of the components) acting on the same substrate.

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Book Reviews

The Physical Chemistry of Electrolytic Solutions. By HERBERT S. HARNED, Professor of Chemistry, and BENTON B. OWEN, Associate Professor of Chemistry, Yale University. (American Chemical Society Monograph Series.) Reinhold Publishing Corporation, 330 West 42nd Street, New York, N. Y., 1943. xi + 611 pp. Illustrated. 15.5×23.5 cm. Price \$10.00.

Every biological system is an electrolyte solution, and life undoubtedly arose in the most widespread of all electrolyte solutions, the ocean. Furthermore, these electrolytes influence profoundly all the other components of the living cell. This is the principal justification for reviewing this comprehensive monograph in this journal, even though the discussion of Harned and Owen makes no explicit mention of biochemistry.

The plan of the work falls into two distinctly different sections. The theory of interionic forces as developed by Debye and Hückel, and later by Onsager and others, is taken as the foundation of the theoretical discussion in the opening chapters. The fundamental thermodynamic relations to be used later are briefly stated in Chapter 1, and the following chapter gives the fundamental concepts of the interionic attraction theory. As the reader is warned in the preface, the treatment in this chapter is highly generalized and abstract. It is distinctly *not* a simple introduction to the fundamental ideas of the theory, except perhaps for the reader with an unusual amount of mathematical background. Most biochemists who are anxious to obtain a grasp of the fundamentals of the theory will find the underlying physical picture more clearly presented in such books as Clark's "Determination of Hydrogen Ions" or MacInnes' "Principles of Electrochemistry" than in the present volume. The third chapter develops the theory of the thermodynamic properties of electrolyte solutions in terms of the interionic attraction theory. It includes a discussion of Bjerrum's theory of ionic association and of the theory of the salting out effect.

Chapter 4 presents the very complex theories that have been developed to describe irreversible processes in electrolyte solutions: these deal with such properties as viscosity, conductivity, diffusion, and the variation of conductance and dielectric constant with frequency; also the effect of very intense electric fields on the properties of strong and weak electrolytes. The mathematical details of the complete theory are so complex that the authors have not attempted to present all the steps in the derivation of the final equations. They have restricted themselves to a discussion of the fundamental concepts, with a general outline of the method of deriving the final equations and a full statement of these equations themselves. This extremely difficult task is, on the whole, well done, although many chemists would have appreciated a fuller portrayal of the underlying physical picture from which all the mathematical treatment is developed.

Chapter 5 is a compilation of a large number of numerical tables, giving the slopes of many characteristic functions appearing in the theory of the properties of electro-

lytes. These are adapted to cover a wide range of temperatures and include data on the dielectric constants of several important solvents which have been used in the study of electrolyte solutions. The reader must remember, when using these tables, that the authors have deliberately chosen not to use the present accepted values of the electronic charge, of Avogadro's number, or of the Boltzmann gas constant. In order to avoid recalculating the data given later in the book, they have used the earlier values for these qualities, which differ from the values accepted as most reliable today by about 0.7 per cent. For the purposes for which the functions in these tables will generally be used, the differences introduced by using the correct values would be trivial. However, anyone who uses the tabulated functions to more than two significant figures should remember the uncertainty thereby introduced. It is to be regretted that the authors did not recalculate these functions in terms of the present accepted values for fundamental constants. It must be admitted, however, that the already prodigious labor that must have been devoted to the preparation of this book would have been greatly increased if the authors had recalculated all their data along these lines.

The rest of the book is devoted to the systematic presentation of experimental data, with the aid of the theories already developed. Details of experimental technique are not given here, except incidentally in a few cases where some word about them is necessary for the understanding of the results.

Chapter 6 presents the most accurate measurements available for conductance, transference numbers, viscosity, and diffusion in electrolyte solutions. The beautiful agreement of the experimental conductance measurements for many simple strong electrolytes with the values predicted by theory is strikingly portrayed, and the deviations from the theory for many other electrolytes—perhaps due to incomplete dissociation—are also very well brought out.

Chapter 7 discusses ion association due to Coulomb forces, particularly in solutions of low dielectric constants, and also the effect of high fields on strong and weak electrolytes.

Chapter 8 considers the thermochemical properties of ionic solutions, their partial molal volumes and coefficients of expansion and compressibility. A great body of experimental data is summarized here. The primary emphasis is on the variation with ionic strength of the properties concerned. The absolute values of such quantities as the partial molal volume or heat capacity in dilute solution, which are so extraordinarily low for electrolytes as compared with most other substances, receive only brief treatment. The adequate discussion of these phenomena would require a further development in terms of a detailed picture of the structure of liquid water and of the compression and orientation of water molecules around the ionic solutes. The theory of these phenomena was treated by Webb and Zwicky, and further developed by Bernal and Fowler and others into a detailed picture of the structure of water and ionic solutions; this is occasionally mentioned in the book but not portrayed in detail. Necessarily such a detailed picture of the structure of any liquid is far from adequate or complete today. The emphasis in Harned and Owen's monograph is on the picture of ions as charged spheres in a continuous medium of a given dielectric constant. Although such a picture is only a first step in our understanding of electrolyte solutions, the great body of data presented in this book gives an im-

pressive idea of the power of this simple picture to explain a wide range of phenomena. The simple theory, as Debye said long ago, works better than it has any right to do.

Chapter 9 covers the calculation of activity and osmotic coefficients from freezing points, boiling points, and vapor pressures.

Chapter 10 presents the thermodynamics of galvanic cells. There is, in this chapter, a considerable discussion of concentration cells with liquid junction, in which the authors have laid great stress on the fact that individual ionic free energies cannot be determined by measurements on systems of this or any other type. Their discussion in this section might perhaps convey an unduly discouraging impression to the beginner concerning the value of measurements on cells with liquid junctions. I could wish that they had laid some emphasis on the observations of Scatchard and Buehrer, and of Hamer, which indicate that uncertainties in the measurements on cells with liquid junction often arise from the heat of mixing of the two solutions at the junction and can be reduced by improved temperature control. The final section of this chapter discusses the standardization of pH measurements, the treatment being based largely on the recent recommendations of MacInnes, of Hitchcock, and their collaborators. The methods by which uncertainties due to liquid junctions may be minimized are here well portrayed, and the recommendations given in this section are sound and useful. Most biochemists in their daily work are not concerned with pH measurements requiring the degree of precision at which the authors aim here. However, it is well to be reminded of the great accuracy to which such methods can attain when they are carried out with due attention to the conditions required by both theory and experiment.

The last five chapters are in the nature of short monographs on different types of electrolytes. Chapter 11 is devoted to hydrochloric acid, probably the most widely and carefully studied of all electrolytes, and important in other connections because the measurements made on cells containing it are essential in evaluating studies on many weak electrolytes. Chapter 12, on strong 1-1 Electrolytes in aqueous solution, contains a collection of very accurate data on activity coefficients, and a discussion of salting out effects and of the application of extended treatments applicable to the behavior of concentrated solutions of strong electrolytes. Chapter 13 treats of polyvalent electrolytes, and Chapter 14 of mixtures of strong electrolytes. The latter chapter discusses many of the important solubility studies of Brönsted and others, which have significant implications for many biological systems as well as for theoretical chemistry. Chapter 15 considers the ionization and thermodynamic properties of weak electrolytes. The most important of all weak electrolytes, water, is discussed in great detail. The ionization constant of water, its heat of ionization, and other related thermodynamic quantities, are given with very high precision and over a wide range of temperatures and pressures. Then follows a treatment of simple weak acids and bases, and of amino acids. This chapter contains a thorough compilation of many data of high accuracy on certain substances of great importance in biochemistry. An appendix of nearly 50 pages includes a large number of supplementary tables too extensive in character to be conveniently included in the text. There is an author index, a thorough subject index, and at the beginning, a very extensive glossary of the symbols employed. I have noted a number of trivial typographical errors, but no serious ones.

This monograph brings together a systematic presentation of theory and a very comprehensive treatment of a great body of experimental data. I believe that its outstanding contribution is in the latter respect. Everyone who needs to obtain the most accurate information on such subjects as activity coefficients in electrolyte solutions, or conductance measurements of high precision, will find the book indispensable. Not only is the best experimental work in these fields brought together here in one place, but it has, in general, been critically evaluated with great care in the light of the long experience of the authors in this field.

JOHN T. EDSALL, Boston, Mass.

Vitamins and Hormones—Advances in Research and Applications, Volume II. Edited by ROBERT S. HARRIS, Associate Professor of Nutritional Biochemistry in the Massachusetts Institute of Technology, and KENNETH V. THIMANN, Associate Professor of Plant Physiology in Harvard University. Academic Press, Inc., New York, N. Y., 1944. xv + 514 pp. Price \$6.80.

The reviews in Volume II cover 11 different subjects; 7 deal with vitamins, 3 with hormones and one with dental caries. The vitamin reviews are well selected since 2 cover water-soluble factors, 2 fat-soluble vitamins, and 3 cover such general topics as vitamins in fat metabolism, vitamins and cancer, and the nutritional requirements of primates other than man.

McHenry and Cornett discuss the role of vitamins in the anabolism of fats by emphasizing the fundamental aspects of fat metabolism and then suggesting the possible relation of specific vitamins to the known reactions. They emphasize two important future fields of research, namely, studies on fundamental biochemical reactions in which the vitamins serve and the importance of the vitamin supplements made to the diets of experimental animals used for fat metabolism.

It is most valuable to find a chapter on biotin prepared by Dr. Melville who has been so closely associated with the work on the chemistry of this compound. All the pertinent information is included and it is especially fortunate that he was able to include the newer work of Kögl which has just recently become available. It is most interesting to note the rapidity with which our knowledge of biotin has developed.

The chapter by Day on the nutritional requirements of primates other than man is very complete and most welcome at this time because no reviews are available on the nutrition of the monkey and because this animal is now being used more extensively in nutritional studies. Considerable space is given to the discussion of vitamin M first described by Day and coworkers, and rightly so, because there is much interest in this and related factors at the present time.

In spite of the fact that the importance of vitamin E in nutrition, especially human nutrition, is still not settled, Dr. Mason has developed a most interesting summary of the newer developments in the field. He suggests that future studies will center around the relation of tocopherols to utilization of lipids, to intermediary metabolism of fats, and to oxidative mechanisms essential to the integrity of muscle and other tissues.

The chapter on the chemistry and physiology of vitamin A is most comprehensive and covers the entire subject from the historical developments to detailed methods

for estimating vitamin A. Much less space is given to the physiology of vitamin A than to its chemistry. The suggestion that the vitamin A value of foods containing carotene must be "written down" to $\frac{1}{2}$ or even $\frac{1}{3}$ the nominal number of International Units due to the carotenoids present may be questioned. It would be better to suggest an increased requirement for vitamin A when carotene containing foods are used.

It is most surprising to the reviewer that Ansbacher found 300 papers dealing with p-aminobenzoic acid. It was only in 1940 that p-aminobenzoic acid was suggested as an essential metabolite associated with bacterial growth and within a few years it has found a place in many phases of biochemistry, nutrition and bacteriology.

The summary on dental caries by Cox is not as general as one would expect to find in *Vitamins and Hormones*. A considerable amount of detail is given on the recording of dental caries and the production of dental caries in the rat, which the average reader is not interested in. About one page is given to nutritional studies and 14 pages to fluorine and dental caries. However, it will be valuable to have this as a reference paper on caries.

One of the first relatively short and critical reviews on vitamins and cancer appears under the authorship of Burk and Winzler. The review covers both the vitamin content of tumor tissues and the effect of restricted and excess amounts of each of the vitamins on the incidence and the growth of tumors.

Dr. Dodds in a very few pages gives a critical evaluation of the relation of hormones and cancer. He states that no clear cut relationship has been shown between any particular endocrine and the growth of tumors but concludes that the most hopeful indications are related to the effect of the estrogens on tumor growth.

In contrast to the chapter on vitamin A, the two remaining chapters on hormones cover very limited fields of work. The chapter on the effect of androgens and estrogens on birds is restricted to the effect of crystalline, characterized substances on the reproductive organs and secondary sexual characteristics. The chapter on "X-Ray Crystallography and Sterol Structure" covers X-ray studies on cholesteryl iodide, sex hormones, heart poisons and sterols. Dr. Crowfoot points out that X-ray studies have not only been especially valuable in studying the structure of the sterol skeleton but gives an example of the application of a new tool in chemical research.

Each chapter has been prepared with great effort and care and much is gained from reading each review, but one cannot help but wonder why the diverse subjects have been included within a single book. However, as the number of volumes in this series increases I imagine we will find that these reviews will fit into a more complete picture of hormones and vitamins.

C. A. ELVEHJEM, Madison, Wisc.

The Metabolism of Fat. By IDA SMEDLEY-MACLEAN, Biochemical Department of the Lister Institute of Preventative Medicine. Methuen and Co. Ltd., London, 1943. vi + 104 pp.

This is a small volume with 94 pages of text giving a very readable summary of modern views on the metabolism of fat and including most of the fatty acid compounds. The standing of the author among the world's biochemists who have inter-

ested themselves in the lipids ensures a good and impartial review, and such is the case. The treatment is brief and to the point. The conclusions reached are sound and are in general in accord with those held by workers in the field.

The text is remarkably free from errors; in fact, the only one the reviewer recognized is the name Folchin for Folch.

The following headings of chapters indicate the subjects treated: Introductory; Method of Synthesis of the Fatty Acids; The Constitution of the Natural Polyene Unsaturated Acids; The Part Played by the Polyene Unsaturated Fatty Acids; The Method of Oxidation of the Fatty Acids; The Method of Combination of the Fatty Acids; Glyceryl and Cholesteryl Esters, Phospholipins, and Galactolipins; The Method of Transport of the Fatty Acids; Bibliography and Index. The organic chemical background of the fatty acid compounds and their transformations is given especial attention.

W. R. BLOOR, Rochester, N. Y.

Advances in Protein Chemistry, Vol. I. Edited by M. L. ANSON, Continental Foods, Hoboken, N. J., and JOHN T. EDSELL, Harvard Medical School, Boston, Mass. Academic Press Inc., New York, New York, 1944. xi + 341 pp. Price \$5.50.

This volume is to initiate an annual publication dealing with protein chemistry. It is indeed a pleasure to welcome such a venture and especially since so high a standard of excellence has been set by this, the first volume of the series. The emphasis in volume one is placed on proteins as they occur in nature, as components of biological system. Chapters have been authoritatively written by nine collaborators.

Erwin Chargaff discusses the lipoproteins. These substances, while of undoubted biological importance, represent a most difficult field of investigation as many researchers can testify. The binding between proteins and lipids can be of a very varied kind. The approach which Chargaff has himself employed and which consists in studying complexes formed between purified proteins and lipids when mixed under controlled conditions, appears to be the most profitable at the present time.

Francis O. Schmitt considers some of the structural proteins in the animal body. In this discussion he deals principally with the nerve proteins, collagen, muscle and fibrin. He relies for the most part on such information as can be obtained through the use of polarized light, X-ray diffraction and the electron microscope. This chapter gives us some indication of the difficulties which biologist and biochemist face if they expect to move forward in the elucidation of the functions of the ultrastructures in biology.

Henry P. Treffers summarizes the chemical aspects of immunology. While immunology holds out promise of utility in the study of protein chemistry, that promise has as yet been fulfilled to a very limited extent. This comment is not intended as a criticism of this particular paper which, incidentally, is excellent. The truth of the matter is that the contribution of protein chemistry to immunology is very apt to be much more substantial than the contribution of immunology to protein chemistry.

David M. Greenberg outlines the experimental methods and conclusions regarding the interaction of the alkalin earth cations with protein. Stress is laid on the calcium ion. The possible methods by which proteins bind calcium are discussed. He concludes that the most probable method of binding is through the free carboxyl and free hydroxyl groups in the protein.

Bacon F. Chow reports on the purification and properties of the protein hormones from the pituitary gland. He also includes a discussion of the chorionic gonadotropic hormone and the gonadotropic hormone from pregnant mare's serum. Emphasis is placed on test of purity by physical methods. Only a limited number of preparations of protein hormones have been found to satisfy the criteria of purity as established by electrophoresis, by ultracentrifugation, and by constant solubility. They are the metakentrins from hog and sheep, lactogenic hormones from oxen and sheep, posterior lobe hormone from oxen, and adrenotropic hormones from sheep and hogs.

Donald S. Payne and L. S. Stuart have a section on Soybean Protein in Human Nutrition. While this article is interesting and informative, the chapter stands apart from the rest of the book and it would have been easy to have selected a more appropriate topic to have filled this space.

Jesse P. Greenstein has a long paper on nucleoproteins. He first discusses the composition and properties of the nucleic acids. Then he considers the interaction of nucleic acids with purified proteins and with amino acids. Finally, he deals with nucleoproteins as they occur in nature. He concludes that the bonds between nucleic acids and proteins are largely electrostatic.

Kenneth Bailey discusses the proteins of skeletal muscle and centers his attention on myosin. To the reviewer this is the most interesting chapter in the book. Here we are coming close to the ultimate function of myosin and how it performs this function. The reviewer feels that there are other and more attractive ways of explaining muscle contraction than by postulating a change of an α -keratin to a super-contracted structure. The essential question to be answered is whether or not muscle contraction is due principally to a change in the internal energy of myosin or to a change in the entropy of myosin. As yet there are no experiments to indicate which is the correct view.

The reading of this book impresses one with the very fragmentary state of our knowledge of proteins as they occur in nature and for the great need for more extensive research on the subject. The volume brings this lack of information and understanding into sharp focus and should prove a powerful stimulus to further work. The editors and collaborators should be congratulated on the service that they have done protein chemistry.

HENRY B. BULL, Chicago, Ill.

Genes and the Man. By BENTLEY GLASS, Associate Professor of Biology, Goucher College. Bureau of Publication, Teachers College, Columbia University, New York, N. Y., 1943. x + 386 pp. Price \$2.80.

Encompassing in a broad sweep the fields of Genetics, Embryology and Physiology, the book is a successful attempt to present to the unprepared mind the biological aspects of the history of human life. Starting with a comprehensive discussion of the morphological and physico-chemical problems of cell division, it then dwells in a pleasantly original manner upon the fundamentals of reproduction and heredity. Ample space is devoted to an analysis of the genetic and physiological basis of sex. Subsequent chapters deal with the interaction of the hereditary pattern with the external environment, and with the realization of the potentialities in the course of development. An exposition of the factors leading to senescence and death concludes the book.

This rather wide issue is treated in a lively and interesting style. Illustrated by diagrams and pictures, the information given is in most respects up-to-date, and well balanced in its dealings with the various viewpoints of the subject-matter. The merits of the author consist not so much in furnishing another textbook on these different fields of Biology, but in welding them together with the purpose of showing what has made man what he is. Certain shortcomings in the presentation of the various problems are thus unavoidable. One might perhaps regret that, while a fully detailed description of the morphological picture of development is given, one finds only occasional side-glances upon the analytical aspects of Embryology and their relation to genetic conceptions. In some other instances, the frame of the book might have suggested a further reduction of the experimental data in favor of a broader discussion of the methodological side of approach. However, as it is, the book is warmly recommended. It will serve teachers of High Schools as a reliable source of information. Its standards may be said to surpass at some points the level which can be expected to be attained by undergraduates.

J. HOLTRETER, Montreal, Canada.

The Living Body. By CHARLES HERBERT BEST, Professor and Head of Department of Physiology, University of Toronto, and NORMAN BURKE TAYLOR, Professor of Physiology, University of Toronto. Henry Holt and Company, New York, N. Y., 1944. xxii + 571 pp. Price \$3.90.

The writing of satisfactory introductory texts in sciences like physiology, which are absolutely dependent upon ancillary sciences such as physics, chemistry and anatomy, is an extremely difficult assignment. Even simple physiological phenomena tend to be obscure without an understanding of the basic sciences. Nevertheless an introduction to physiological knowledge is very much worth while for students who have not and will not become proficient in the ancillary sciences. Consequently it becomes essential that text-books be available which do not presuppose an extensive scientific background but yet expound the basic principles and some of the details of current knowledge of function in living systems in an accurate and readable way.

Teaching physiology to elementary and advanced students for more than a score of years has taught the reviewer to be charitable of the attempts of others to accomplish what he recognizes he has never satisfactorily achieved in the way of presenting a coherent, balanced and well-grounded presentation of the subject for beginning students. It is the opinion of the reviewer that most teachers of introductory courses in physiology attempt to include very much too many facts in their presentations and that their students as a consequence ordinarily fail to grasp broader concepts. Elementary teaching in such a science as physiology is essentially a succession of compromises between the objectives of thoroughness and extensiveness. No simple formula is available to lead the teacher in his choices in this matter.

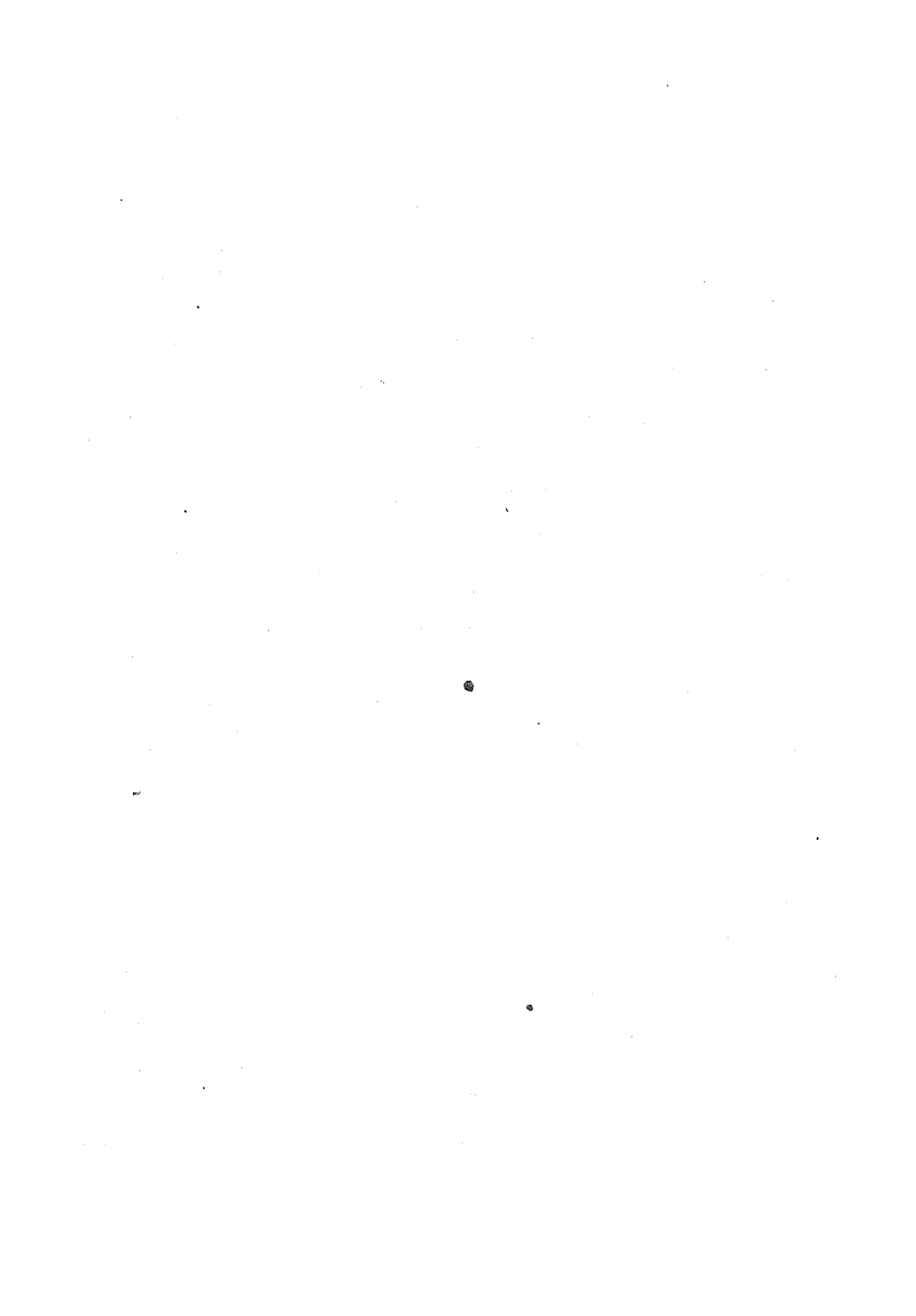
The revised edition of the *Living Body* by Best and Taylor is an admirable example of such a compromise. It is necessarily imperfect but in the opinion of the reviewer it should be useful in the instruction of students of nursing, medical technology and home economics. It is likely that such items as Plate I on the morphology of blood cells and Figure 37 illustrating the chloride shift, among others, will not be appreciated by beginning students except as inspirational material. Nevertheless

such material quite beyond their depths may be worth inserting to stimulate the interest of superior students.

The reviewer has been unable to determine what principle the authors followed in determining what anatomical material should be included and what omitted. In general histology has been stressed, in accord with British tradition, but the total amount of anatomy included will not meet the needs of nursing students in that science. The conventional American teaching program is not served especially well by so much admixture of histology and physiology because in general the students in elementary physiology courses have previously studied general biology or zoology and have thus become at least superficially acquainted with much that has been presented on the morphological side in this book. The reviewer would prefer to see either more or less attention devoted to morphology, the latter if the book is to be used with students having a background in anatomy, and the former if used as a complete text book in human biology. Particularly, there is a lack of material on the anatomy of muscles, tendons, bones and joints, and of the viscera, for use as a text in human biology.

On the whole the treatment is simple and clear and the book should assist teachers in improving what is today a deplorable situation, the gross ignorance of so many college-trained persons in the fundamentals of human biology. The authors deserve the appreciation of teachers for their painstaking efforts.

MAURICE B. VISSCHER, Minneapolis, Minnesota.



The Leucine, Valine, and Isoleucine Content of Meats *

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INTRODUCTION

Microbiological methods for the estimation of several amino acids have been described recently (McMahan and Snell (1), Kuiken, *et al.* (2), Shankman (3), and Hegsted (4)). Similar methods of analysis have also been adapted to the direct determination of leucine and valine in animal tissues (Schweigert, *et al.*, 5). These new approaches have afforded easier and more simplified methods for the determination of amino acids and are even more important where satisfactory chemical methods are not available (for example, leucine, valine, isoleucine).

In this paper values for the valine, leucine, and isoleucine content of meats and the retentions of these amino acids during cooking are reported.

EXPERIMENTAL

Since the publication of a detailed assay procedure for leucine and valine (5) a similar assay procedure has been developed for isoleucine. The components of the medium, concentration of constituents and techniques of assay are the same as for leucine and valine with the appropriate amino acid omitted from the basal medium. A *DL*-isoleucine mixture was used as the standard and the following levels were used for producing the standard curve: 0, 25, 50, 75, 100, 125, 150, 200, and 250 μ g. per tube. The entire contents of each tube after an incubation period of 72 hours were titrated with 0.1 *N* NaOH. A typical standard curve showing the relationship between the level of amino acid and alkali titration is shown in Fig. 1.

Several methods for the hydrolysis of the animal tissues were tested, and maximum liberation of isoleucine was obtained by the following procedures: refluxing for 24 hours with either 5 *N* H₂SO₄ or 5 *N* HCl, autoclaving for 5-10 hours with either

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5 *N* H₂SO₄ or 2 *N* HCl. Autoclaving with 2 *N* HCl for 5 hours was used for all subsequent assays. The stability of isoleucine to these methods of hydrolysis was followed by recoveries of added isoleucine to the samples before treatment.

Since a *dl*-isoleucine standard was used, it was necessary to establish as far as possible the purity of the compound used. Three different *dl*-isoleucine samples gave variable responses to *L. arabinosus*. Two of these samples, A and B, which had lower

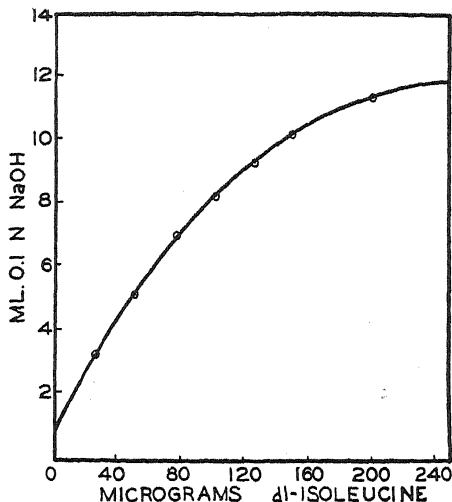


FIG. 1

Typical *dl*-Isoleucine Standard Curve

This curve is obtained by plotting the number of ml. of 0.1 *N* NaOH obtained at each level of amino acid indicated. Triplicate or duplicate tubes are used at each level of amino acid standard. The amino acid content of samples is readily determined by interpolation of the standard curve.

activity than sample C, were tested for moisture and no loss in weight occurred. A sample of *dl*-isoleucine¹ (at least 97 per cent pure) was compared with sample C to test the reliability of sample C as a standard. The high state of purity of sample C was verified, since identical standard curves were obtained. Solutions of the *dl*-isomer are stable, and no change in activity was observed after several months as compared to a freshly prepared standard. Sample C was used as the standard in all later assays, and all results are calculated on the basis of this standard.

Since the water soluble constituents are usually discarded when purified proteins are prepared from natural foodstuffs (Beach, *et al.*, 6), the possible losses of leucine,

¹ We are indebted to Dr. R. T. Major of Merck and Co., Rahway, New Jersey, for this preparation. By their tests this sample was found to be at least 97 per cent pure.

valine, and isoleucine due to water extraction were investigated. Two cooked meats (roast veal and roast lamb) were used for this study. The same procedure as outlined by Beach and co-workers was used for extraction of the water-soluble constituents after the samples were dried and ether-extracted. The water extracts thus obtained were analyzed, and the per cent of each amino acid found in these extracts is shown in Table I.

TABLE I

*The Losses of Valine, Leucine, and Isoleucine Due to Water Extraction
in the Preparation of Purified Proteins*

Amino acid Sample	Valine		Leucine		Isoleucine	
	Roast veal	Roast lamb	Roast veal	Roast lamb	Roast veal	Roast lamb
Weight of sample (g.)	100	100	100	100	100	100
Per cent amino acid in meat	1.55	1.17	2.15	2.22	1.76	1.59
Total mg. of amino acid in sample	1550	1170	2150	2200	1760	1590
Total mg. in water extract	48	28	77	45	35	16
Per cent of the total amino acid lost by extraction	3.1	2.4	3.6	2.0	2.0	1.1

The methods of preparation, cooking and curing of the meat samples used for amino acid analysis have been previously reported, Schweigert, *et al.* (7), McIntire, *et al.* (8, 9). These samples were stored in a -4°C . cold room and removed only when portions were weighed for analysis. The percentages of leucine, valine, and

TABLE II

Detailed Analysis of Meats

	Protein	Valine in meat	Valine in protein	Leucine in meat	Leucine in protein	Iso- leucine in meat	Iso- leucine in protein
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
<i>Veal</i>							
Leg	22.1	1.17	5.3	1.50	6.8	1.17	5.3
Roast leg	30.6	1.55	5.1	2.15	7.0	1.76	5.7
Sirloin	20.6	1.18	5.7	1.26	6.1	1.30	6.3
Braised sirloin	30.7	1.67	5.5	2.00	6.5	1.86	6.1
Shoulder	19.2	0.97	5.1	1.55	8.1	1.05	5.5
Roast shoulder	29.2	1.32	4.5	2.38	8.2	1.66	5.7
Shoulder chop	21.1	1.13	5.4	1.65	7.8	1.14	5.4
Braised chop	31.5	1.73	5.5	2.28	7.2	1.69	5.4
Stew meat	22.0	1.12	5.1	1.73	7.9	1.17	5.3
Cooked stew	31.8	1.84	5.8	2.85	9.0	1.81	5.7

TABLE II—Continued

	Protein per cent	Valine in meat per cent	Valine in protein per cent	Leucine in meat per cent	Leucine in protein per cent	Iso- leucine in meat per cent	Iso- leucine in protein per cent
<i>Lamb</i>							
Leg	18.5	0.92	5.0	1.54	8.3	1.13	6.1
Roast leg	26.2	1.25	4.8	2.12	8.1	1.55	5.9
Sirloin	16.4	0.75	4.6	1.30	7.9	0.91	5.5
Broiled sirloin	26.6	1.18	4.4	2.14	8.1	1.47	5.5
Stew meat	18.6	0.91	4.9	1.51	8.1	1.12	6.0
Cooked stew	28.4	1.44	5.1	2.28	8.0	1.69	6.0
Leg	18.6	0.86	4.6	1.49	8.0	1.08	5.8
Roast leg	26.8	1.17	4.4	2.22	8.3	1.59	5.9
Sirloin	16.6	0.76	4.6	1.31	7.9	1.02	6.1
Broiled sirloin	22.1	1.00	4.5	1.77	8.0	1.22	5.5
Stew meat	17.5	0.88	5.0	1.46	8.3	1.10	6.3
Cooked stew	27.7	1.28	4.6	2.24	8.1	1.57	5.7
<i>Pork</i>							
Ham	16.3	0.86	5.3	1.18	7.3	1.03	6.3
Cured ham	15.6	0.83	5.3	1.15	7.4	0.94	6.0
Ham	13.0	0.69	5.3	0.92	7.1	0.81	6.2
Cured ham	12.6	0.70	5.6	0.94	7.5	0.77	6.1
Ham	16.0	0.86	5.4	1.19	7.4	0.87	5.5
Cured ham	15.1	0.84	5.6	1.15	7.6	0.89	5.9
Ham	14.9	0.76	5.1	1.05	7.1	0.81	5.4
Cured ham	14.6	0.76	5.2	1.06	7.3	0.86	5.9
<i>Beef</i>							
Rib	17.7	0.87	4.9	1.34	7.6	1.03	5.8
Round	20.4	1.05	5.2	1.60	7.9	1.21	5.9
Beef liver	19.8	1.26	6.4	1.63	8.2	1.12	5.7
Beef liver	20.0	1.19	6.0	1.72	8.6	1.11	5.5
Beef tongue	16.0	0.79	5.0	1.23	7.7	0.92	5.7
Beef heart	16.1	1.01	6.3	1.35	8.4	0.84	5.2
Beef kidney	14.5	0.79	5.5	1.12	7.7	0.78	5.4
Beef kidney	14.6	0.76	5.2	1.17	8.0	0.81	5.6
Beef brain	11.3	0.50	4.4	0.80	7.1	0.52	4.6
Beef brain	10.2	0.52	5.1	0.78	7.7	0.57	5.5
Veal liver	17.1	0.95	5.6	1.33	7.8	0.94	5.5

isoleucine in samples of fresh pork, veal, beef, and lamb muscle meats, and beef organ meats are shown in Table II. The percentages of these amino acids in the protein are also shown in Table II. All values listed are calculated on the basis of 100 per cent activity for the *l*-isomer and 0 per cent for the *d*-isomer; thus 50 per cent activity for the *dl* mixture.

The retention of leucine, valine, and isoleucine during cooking was calculated on the basis of the weight change during cooking as outlined by Schweigert, *et al.* (10). A few drippings were available for amino acid analysis but less than 2 per cent of the total amino acid content of the cooked meat could be accounted for in the drippings. The results on cooking veal and lamb cuts and curing pork are shown in Table III.

TABLE III

Per Cent Retention of Amino Acids during Cooking Veal and Lamb and Curing Pork

Type of cooking	Valine	Leucine	Isoleucine
<i>Veal</i>			
Roast leg	93	99	91
Braised sirloin chop	86	96	91
Roast shoulder	93	100	106
Braised shoulder chop	95	86	92
Stew	101	101	98
<i>Lamb</i> (average of 2 series)			
Roast leg	95	101	101
Broiled sirloin chop	99	103	97
Stew	99	99	95
<i>Pork</i> (average of 4 series)			
Cured ham	97	97	96

DISCUSSION

The standard curves obtained for isoleucine on the synthetic medium with *L. arabinosus* used as the test organism, are similar to those obtained for leucine and valine on a similar medium. The methods of assay and sample preparation most satisfactory also parallel the leucine and valine results. The effect of time on the liberation of isoleucine was determined when the tissues were hydrolyzed with 2 *N* HCl. A rapid increase in isoleucine values was obtained up to 3 hours, with a slight increase up to 5 hours, but no further difference in results was observed with 8 or 10 hours of treatment. Fifteen recoveries of isoleucine ranged from 90 to 114 per cent, averaging 99 per cent. Leucine and valine recoveries have been reported previously, Schweigert, *et al.* (5).

The 97 per cent pure *dl*-isoleucine and sample C when compared simultaneously, exhibited the same specificity to *Lactobacillus ara-*

binosus. Although this is not absolute proof that 50 per cent activity was obtained (on the basis of 100 per cent activity for the *l*-isomer and 0 per cent activity for the *d*-isomer), it is good evidence that a very nearly pure sample had been obtained. A *l*-isomer was not available, therefore a direct comparison of the *l*- and *dl*-isomers could not be made. The results on valine and leucine isomers as amino acid standards have been reported earlier. The necessity of a reliable standard for any amino acid determination should be emphasized.

The amounts of valine, leucine, and isoleucine extracted by boiling water during the preparation of purified proteins are small, ranging from 1.1 to 3.6 per cent (Table I). Since in the chemical procedures the material which is hydrolyzed prior to amino acid analysis is the isolated protein, the amount of any amino acid removed by water extraction in the preparation of that purified protein is not measured or cannot be accounted for by chemical methods. It is interesting, however, that measurable amounts of soluble amino acids or amino acid complexes can be removed by simple water extraction of the dried, ether-extracted meat. Perhaps other amino acids may be extracted in greater quantities from meat and also the amounts extracted may be more significant when a similar procedure is used to prepare purified proteins from other materials where the proteins or protein fractions are more readily removed by water extraction.

All values in Table II were determined directly on the fresh meat, and the percentage of each amino acid in the meat protein was calculated from the data obtained on the fresh samples and protein determinations (Kjeldahl method). The leucine, valine, and isoleucine content of animal tissue protein averaged 7.7 per cent, 5.2 per cent, and 5.7 per cent, respectively.

The uniformity of the leucine, valine, and isoleucine composition of the animal tissue protein, as shown in Table II, supports the work of Beach, *et al.* (6). They showed that the arginine, histidine, lysine, tyrosine, tryptophan, phenylalanine, serine, threonine, cystine, and methionine contents of muscle protein are remarkably similar. Since a wide variation in physiological function and activity exists between skeletal muscle, brain, liver, kidney, and heart proteins, it is interesting that the amino acid content of the total proteins is nearly the same.

The higher percentages of amino acids in the cooked meat are due merely to the loss in weight of the meat during cooking. Of the fresh tissues, liver, veal, and beef muscle tissues are the best sources of these

amino acids, since the amino acid composition of meats varies with the protein content as indicated in Table II.

Leucine, valine, and isoleucine retention during cooking ranged from 86 to 106 per cent averaging 97 per cent. Eleven cooking series and 4 curing tests were carried out in this study. The high retentions of these three amino acids after cooking and curing meat are in contrast to destruction of certain of the B vitamins observed during similar treatments. Although large amounts of thiamine, nicotinic acid, and riboflavin are found in the drippings particularly after stewing and braising, less than 2 per cent of the leucine, valine, or isoleucine can be accounted for in the drippings.

Beach, *et al.* (6) cooked the meat samples prior to amino acid analysis of the purified proteins from these cooked meats. The present investigation shows that at least in the case of leucine, valine, and isoleucine, the amino acid composition of the original fresh meat could be determined quite accurately from the analysis of cooked meat since an average of 97 per cent of these amino acids are retained in the meat after cooking. Therefore, the leucine, valine, and isoleucine content of cooked meat could be easily calculated from the weight changes during cooking on the basis of the amino acid content of the uncooked meat.

SUMMARY

1. A satisfactory microbiological method for the direct determination of the isoleucine content of animal tissues has been devised.

2. From 1.1 to 3.6 per cent of the valine, leucine, and isoleucine present in the original meat is lost by water extraction, a technique used in the preparation of purified proteins from crude materials.

3. The valine, leucine, and isoleucine content of beef, veal, lamb, and pork muscle meats and beef organ tissues and tissue proteins have been determined. The amounts of these amino acids are relatively constant in the meat protein, averaging 7.7 per cent for leucine, 5.2 per cent for valine, and 5.7 per cent for isoleucine.

4. The stability of valine, leucine, and isoleucine to cooking and curing procedures was studied; and from 86 to 106 per cent retention was observed. Less than 2 per cent of any of these amino acids was found in the drippings.

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Survey of Growth and Gas Production of Genetic Variants of *Saccharomyces Cerevisiae* on Different Sugars *

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INTRODUCTION

The isolation of over 1450 genetically different strains of *Saccharomyces cerevisiae* provided material for the study of the genetic basis of various biochemical characteristics. Previous publications (Lindegren and Lindegren, 1943a, b, c) have dealt with the techniques employed in strain isolation. The pedigree of the "D" family and the volume of growth of its haploid segregants, variants from these, and diploid recombinations on a standard medium have already been described. The present paper considers additional characteristics of the different members of this and other families.

Before selecting physiologically interesting types for intensive study it was necessary to characterize the group as a whole by its mean physiological behavior under standard conditions. With these data at hand, the relative significance of deviations in individual strains could be evaluated. Since the number of strains to be examined was large, it was important that the physiological examination be simple and rapid. After some preliminary experiments, it was decided to measure the volume of growth and the gas production on various sugars either singly or in combination. In addition to the simplicity of the measurements involved, there are two other reasons for selecting these characters for study. Firstly, the preliminary experiments revealed a wide variation in the behavior of the different strains on the same medium as well as of the same strain on different media. Secondly, it seemed

* This work was aided by a grant from Anheuser-Busch, Inc., St. Louis.

probable that growth and gas production were more likely to characterize the physiology of a particular strain than any other two measurements. The desirability of this kind of analysis was further emphasized by certain findings reported briefly in the study of the adaptive enzyme, galactozymase (Spiegelman, Lindegren, and Hedgecock, 1944; Spiegelman and Lindegren, 1944). It was indicated in these studies that two strains originating from the same parent strain could possess strikingly different methods of utilizing the same sugar, one fermenting it, the other oxidizing it by a purely aerobic process.

The existence of non-fermentative and other atypical oxidations of carbohydrates has been demonstrated in other forms with various sugars as substrates. Nord and his co-workers (1939) have made a careful examination of carbohydrate fermentation in *Fusaria* and determined also the low amount of organic phosphorus donors present. They found that in the initial period, fermentation was effected without previous phosphorylation, whereas following this period, fermentation proceeded *via* a phosphorylating mechanism. Trautwein and Weigand (1931) have shown the direct oxidation of maltose by certain molds, and Nord and Engel (1938) have demonstrated its direct fermentation by *Fusarium lini* Bolley. Using the same form, O'Connor's (1940) experiments indicate that the fermentation of trehalose need not involve either esterification by inorganic phosphate or preliminary hydrolysis. Barron and Friedemann (1941) have demonstrated the oxidation of glucose and hexose phosphates by bacteria which do not ferment glucose. This mechanism is also prevalent amongst certain yeasts, particularly the *Endomycetaceae*, some of which cannot ferment any of the carbohydrates (Stelling-Dekker, 1931).

In view of these findings it seemed of value to examine the range of fermentative capacity amongst the genotypes derivable from a single parent. The progeny from 12 strains of bakers' yeasts was examined. The progeny of only six of the strains will be considered here in detail. They are representative of the range of biochemical heterogeneity found in the fermentative characteristics in all twelve. As will be indicated at the proper point, some of the studies reported here were made on random sample strains obtained from all 12 pedigrees.

METHODS AND MATERIALS

A. Yeast Strains. All strains reported on, originated from 12 diploid yeasts. Those symbolized by letters (G, M, FD, F, D, L, BE, HD) stem from commercial baking yeasts collected on the market, whereas strains 800, 801, 806, and 812 were obtained from the yeast collection of the Northern Regional Research Laboratory at Peoria, Illinois, through the courtesy of Dr. L. J. Wickerham.

The following procedure was used to obtain different strains from each of the original cultures. After the culture had been induced to produce spores, four-spored

asci were selected and dissected; the four spores were planted separately on agar. The colonies thus developed give rise to haploid strains which are segregants of the diploid from which they came. Diploid strains were obtained by permitting copulation to occur between the haploids within the ascus and isolating the resulting diploid cells.

B. Media. The basic medium consisted of 0.5 per cent peptone, *M*/15 phosphate buffer adjusted to pH 6.4, and 2 cc. of liquid yeast extract per liter. Carbohydrate was added to the above to make a 2 per cent solution. In the case of the medium labeled "broth," dextrose, sucrose, and maltose were used in a ratio of 2:1:1. Results obtained on single sugars are labeled in the figures with the name of the sugar used. Growth and gas evolution characteristics were examined on sucrose, glucose, galactose, levulose, and maltose.

Measurements

Cultures were inoculated into 20 cc. of medium contained in 25×200 mm. test tubes. Each tube contained an inverted gas tube 11 cm. long with a capacity of 4 cc. The openings of the gas tubes were cut off at a slant to prevent a seal forming at the base. The gas evolved was read at the end of 24 hours by measuring the length of the gas column to the nearest 0.5 cm. The results on gas volume were recorded and plotted in terms of these arbitrary units, each of which is equivalent to 0.36 cc. After 48 hours, growth was determined. The contents of each test tube were agitated to attain uniform distribution of yeast cells and a 10 cc. sample removed and placed in a graduated Hopkins vaccine tube. This was then centrifuged for 20 minutes at 2,000 r.p.m. and the yield read off in terms of arbitrary units of yeast volume. Each such unit represents 0.012 cc. of yeast volume per 10 cc. of culture medium. These arbitrary units were employed in the various plots used in comparing the different strains.

RESULTS

All the results are summarized by spot diagrams which give a pictorial survey of the distribution of the various physiological types. The position of each point characterizes a particular strain.

Gas Evolved Versus Growth

Figs. 1-6 inclusive give the gas yield data obtained when the strains were inoculated into the broth medium containing glucose, sucrose, and maltose. All the data are plotted in terms of the arbitrary units of gas and yeast volume described under "Measurements." Only 1 strain derived from D, falls much below average yield in this mixture. By far the vast majority of the strains from all sources can apparently utilize at least one of the sugars to support growth. With a few exceptions yields of 4.5 and over were obtained. While the yields under these conditions are restricted in the main to a relatively narrow region lying between 4.5 and 7.0 the gas measurements are spread over the

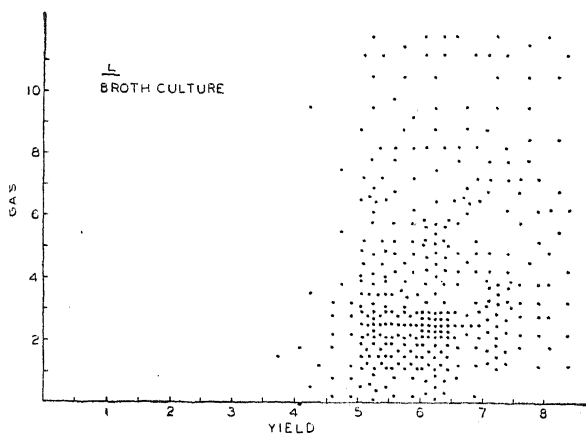


FIG. 1

Biochemical Types in Family L Growing on "3-Sugar Broth"
Each spot represents a different strain.

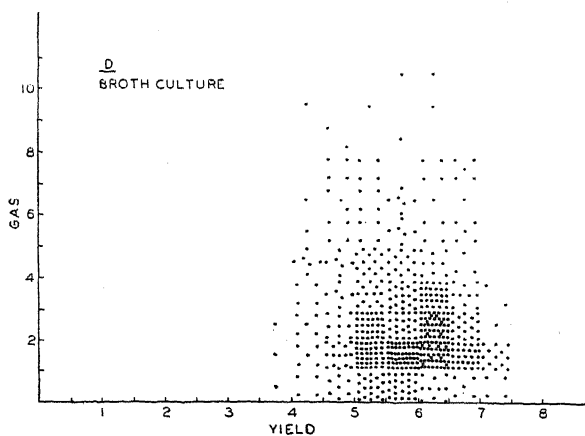


FIG. 2

Biochemical Types in Family D Growing on "3-Sugar Broth"
Each spot represents a different strain.

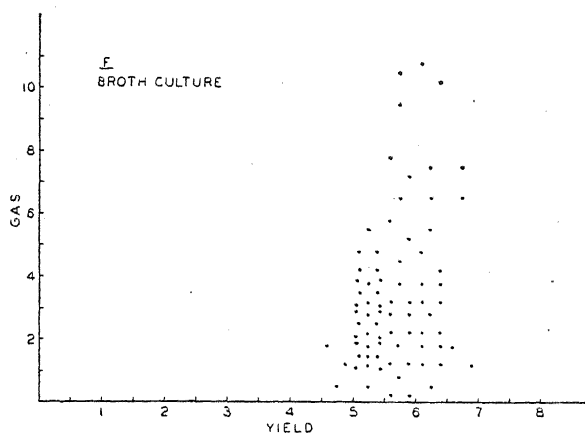


FIG. 3

Biochemical Types in Family F Growing on "3-Sugar Broth"
Each spot represents a different strain.

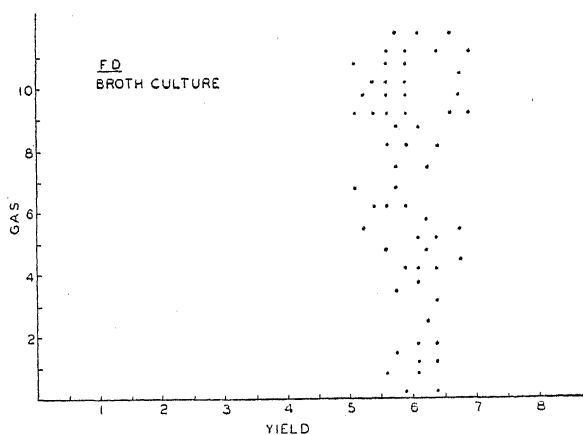


FIG. 4

Biochemical Types in Family FD Growing on "3-Sugar Broth"
Each spot represents a different strain.

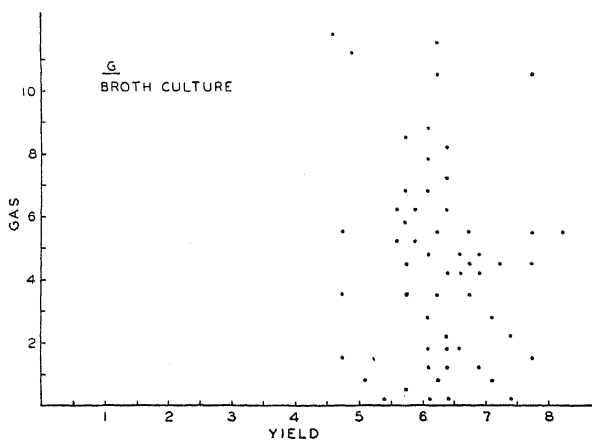


FIG. 5

Biochemical Types in Family G Growing on "3-Sugar Broth"
Each spot represents a different strain.

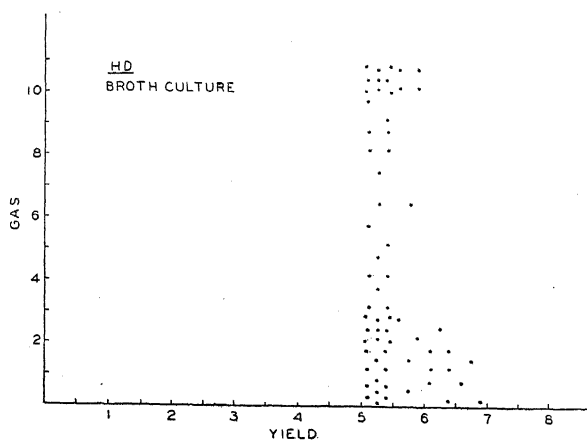


FIG. 6

Biochemical Types in Family HD Growing on "3-Sugar Broth"
Each spot represents a different strain.

entire range of measurements. In all the pedigrees examined there exist some members with relatively high yields and poor (between 0 and 0.5) gas production. It is evident that these strains cannot be metabolizing the sugars present in the same way as those which, though giving the same yield, produce 10–20 times as much CO_2 . In so far as the general distribution is concerned, there is no apparent difference in the physiological types derivable from the original twelve diploids.

Figs. 7–9 inclusive show similar data for growth and gas evolution on single sugars. In these and all subsequent diagrams the strains represented in each figure do not all belong to the same pedigree. Sample strains were selected at random from the twelve pedigrees and these were used in the single sugar experiments. Studies similar to those depicted in Figs. 7–9 were also made with galactose and maltose and showed the same general distribution of points.

It is evident from the figures that the same independence of yield and gas production exists for single sugars as was found in the three-sugar broth medium. One striking difference appears, however, in the wide spread along the yield axis in Figs. 7–9 as compared with that observed in Figs. 1–6. This is due to the fact that there are relatively few yeast strains which cannot metabolize any of the three sugars used in the 'broth' medium, whereas a fair number can be found which find it difficult to grow well on any particular sugar. It will be noted that for all sugars examined both vigorous and poor gas producers were

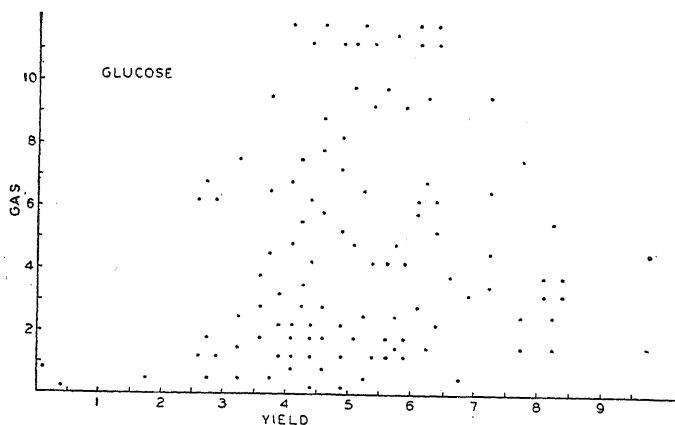


FIG. 7

Representative Strains from All 12 Progenies Growing on Glucose Broth

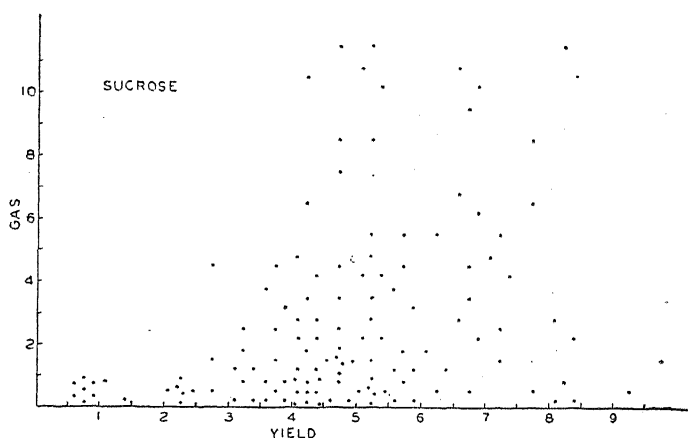


FIG. 8

Representative Strains from All 12 Progenies Growing on Sucrose Broth

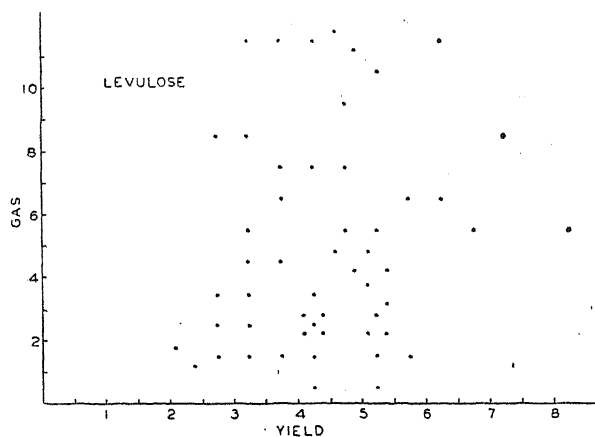


FIG. 9

Representative Strains from All 12 Progenies Growing on Levulose Broth

found among the comparatively high yielding cultures. Here again it is difficult to escape the conclusion that a strikingly different physiology exists as far as carbohydrate utilization is concerned amongst closely related types.

It was of interest to compare more or less directly the yield and gas production of the same strains on different sugars. Figs. 10-12 inclusive give such comparisons for gas production. In these diagrams both coordinate axes represent units of gas produced, the abscissae corresponding to behavior on one sugar, the ordinate being used for the other. The relative distances of any given point from the two axes denote then the comparative behavior of the corresponding strain on the two carbohydrates being compared. Points lying on or close to the

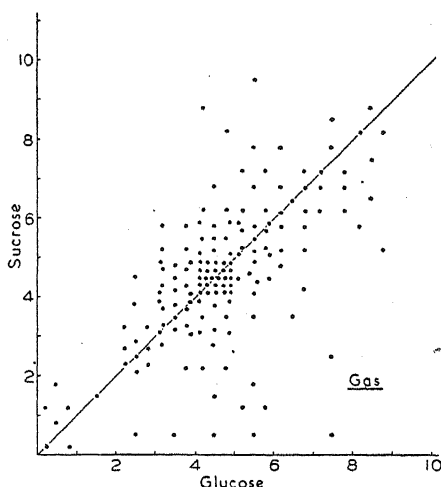


FIG. 10

Comparison of Gas Production on Sucrose and Glucose

45 degree line drawn in each diagram correspond to strains which can handle the two sugars equally well.

As is illustrated by the even distribution about the 45 degree line, Fig. 10 shows an expected general equivalence between sucrose and glucose. There are, however, a small number of strains unable to ferment sucrose successfully although they have no difficulty with glucose. This is probably due to a comparatively low content of the sucrose splitting enzyme in the cells of these strains. Several strains demonstrate the interesting biochemical character of fermenting sucrose significantly better than glucose. One in particular producing 8.3 units on sucrose and only 4.1 on glucose.

The plot of sucrose against levulose given in Fig. 11 yields the same general picture as was obtained in the comparison of the sucrose against glucose (Fig. 10). It is of interest to note that here also strains are found that can ferment sucrose significantly better. Thus one strain produces 8.2 units on sucrose and 2.2 units on levulose.

A comparison of the two hexoses, glucose and levulose, given in Fig. 12 reveals a small but definite shift toward the glucose side. This

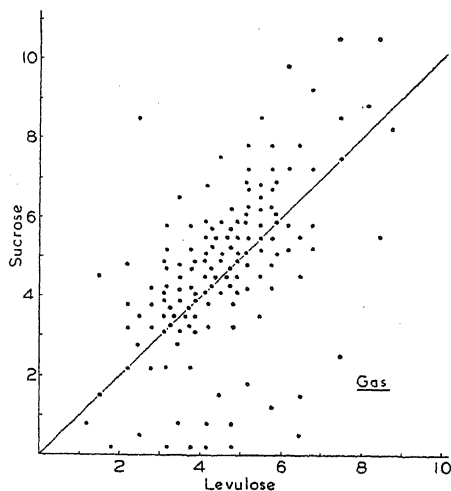


FIG. 11

Comparison of Gas Production on Sucrose and Levulose

is an interesting finding in view of concepts of glucose metabolism by yeast juices through the fructose phosphates. As can be seen, certain strains demonstrate this preference for glucose in an exaggerated manner. One strain produced 7.6 units of gas on glucose and only 2.1 on levulose.

Yield spot diagrams were also made for the same strains growing on the same sugars. Except for the minor movement of certain strains the pairwise comparison of the growths on the different sugars exhibited exactly the same patterns as the corresponding gas diagrams. For the purposes of this presentation a detailed discussion of the yield distribution would add little that is new to the information already obtained from the analysis of the gas diagrams.

Comparisons similar to those on glucose, levulose, and sucrose, given in Figs. 10-12 were also carried out with both galactose and maltose. The spot diagrams will not be given but since certain interesting biochemical types were noted the results may be briefly summarized.

When behavior on glucose was compared with galactose, it was found as was to be expected that the vast majority of strains fell on the glucose side of the 45 degree line. Out of 150 strains examined only

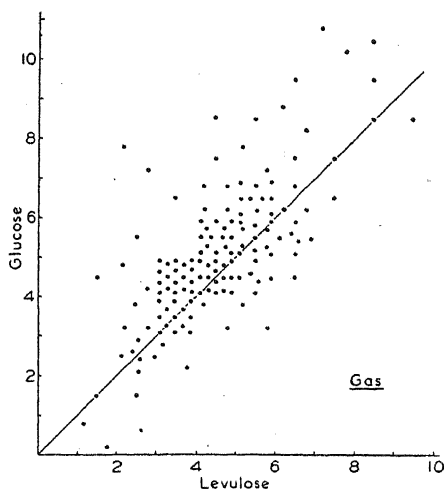


FIG. 12

Comparison of Gas Production on Glucose and Levulose

one showed marked preference for galactose. This particular strain produced 5.7 units of gas on galactose and only 1.4 on glucose. The comparison of galactose against levulose showed a decided preference for levulose in the vast majority of strains. Four strains were however found that showed a significantly better fermentative capacity when galactose was the substrate as compared to that measured on the levulose medium.

When gas production on maltose was compared with that on glucose, it was found the majority of strains could ferment glucose with greater ease. Five of the 150 strains tested were unable either to split maltose or to use it directly in some other fashion. As in the case of the glucose-sucrose comparisons (Fig. 10) several biochemically anomalous strains

were found. For example, one strain produced only 1.2 units of gas on glucose and 5.3 units on maltose. This would be difficult to understand if this strain metabolized maltose by first splitting it into two glucose molecules.

In the comparison of maltose *versus* levulose, the tendency was toward better fermentation of levulose in 82 per cent of the strains tested. This differs from the sucrose-levulose comparison (Fig. 11) in which there exists a slight preponderance on the sucrose side of the 45 degree line. When the two disaccharides, maltose and sucrose, were compared, it was found that 87 per cent of the strains fermented sucrose with greater ease. However, here as elsewhere a wide divergence of biochemical types was found. Seven strains could not ferment sucrose at all but had little difficulty with maltose.

DISCUSSION

For a proper evaluation of the data presented it is important to realize that many of the strains examined in the present study are haploid. The range of physiological behavior exhibited by any family is thus conditioned not only by the types obtainable by segregation and recombination of the segregant from the original parent diploid, but is also dependent on the mutational potentialities of the haploid strains studied. Despite the additional flexibility attained through haploid variation, it is perhaps a little surprising that such a wide variety of physiological types are found within a given family.

Meyerhof (1925) compared the physiology of various yeasts and found that they differed markedly in their metabolic characteristics. However, in the case of his investigation the comparison was not between members of the same family. Highly selected brewing and wine yeasts were compared with the wild type *torulae*. In the present study the twelve original strains were commercial bakers' yeasts. They were thus the result of intensive selection toward a particular physiological property, namely aerobic CO₂ production on sucrose. Despite intense selection they still possess the potentiality for giving rise to strains possessing almost diametrically opposed behavior with respect to the very physiological character for which they were selected.

These findings have implications for studies of the biochemistry of yeast strains. Any experimental procedure which permits sporulation to occur with the consequent introduction of the haplophase may well

give rise to widely divergent results on what may have been considered the same strain. In this connection, it may be of interest to note that heavy sporulation does occur on the surface of most package yeasts sold commercially. Reproducibility of physiological measurements on a strain can be attained in general only if it is diploid and is transferred sufficiently frequently to avoid sporulation. In the course of the investigation several haploid strains were found which had become sufficiently stabilized genetically to be studied.

Although the variability observed, if not controlled, prevents an adequate description of strain physiology, it can serve as a useful tool for the isolation of biochemically interesting types. Several such have already been noted in the discussion of the figures. A comparative study of the carbohydrate utilization of these strains is being made to throw some light on adaptive enzyme formation toward various sugars.

The occurrence of a purely aerobic mechanism for the metabolism of both glucose and galactose in strains which are unable to ferment these sugars has already been noted. Examination of the yield-gas diagrams on single sugars shows that this phenomenon is widespread amongst strains of *S. cerevisiae* and was found in all the pedigrees studied. A comparative study of these strains is being undertaken.

ACKNOWLEDGMENT

The authors would like to express their appreciation for the assistance of Grace S. Colowick in performing some of the experiments reported here.

SUMMARY

1. A comparative study of growth and gas production on 1450 genetically different strains of *Saccharomyces cerevisiae* on 5 different sugars has been made.
2. A wide variety of physiological types was found within the members of every family. The range of variation was as great in highly selected commercial strains as in laboratory strains.
3. Certain biochemical types were pointed out. Among these are strains that produce more gas and give greater yield on maltose than on an equivalent amount of glucose. Analogous strains were found for sucrose. Certain strains have a greater fermentative capacity and yield on glucose than they do on levulose. Others could not ferment glucose appreciably but grew very well on it. Analogous strains were found

for all the other sugars tested, namely, galactose, levulose, maltose, and sucrose.

4. The significance of the results for the comparative biochemical study of the yeasts is discussed.

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Significance of Antonoff's Law in the Studies of Liquid Systems.—Its Applications in the Field of Biological Science

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I. INTRODUCTION

In colloidal or semi fluid systems of various types one can often observe changes of properties with time, usually termed "aging" which indicate that they are not always in a state of equilibrium. They also follow changes of concentration and temperature, sometimes extremely slowly, and thus the only way to discover whether the systems are in equilibrium is to keep them under observation for a considerable length of time. The evidence brought out by F. F. Nord and collaborators indicates that these changes are attributable to processes of aggregation or disaggregation which may be reversible or irreversible. In some cases these changes are accompanied by structural changes as evidenced by adsorption spectra, while in others this is not the case (1). Examples of these will be discussed in the course of this paper.

The systems mentioned are often highly viscous, and one may therefore think that these phenomena are due to high viscosity coefficient. It will be shown in this paper that these characteristics can be found also in ordinary liquids, where one suspects them least of all in view of their high degree of fluidity, and where the dependence of physical properties on the previous history of the liquid can be made apparent.

These phenomena cannot be understood in the light of current theories because these theories do not make any allowance for them. For this reason the author, as a result of his own work on liquids, was led to a new theory of liquids briefly stated in the next paragraph, which makes it possible to interpret the peculiar phenomena mentioned above.

The experimental facts which led to this theory were:

Changes in properties of liquid systems with time can be readily observed, in many cases, if one measures the surface tensions. The experimental determination of surface tensions involves also density measurements.

After measuring the surface tensions of some liquid systems, the author observed variations with passage of time which could not be accounted for easily. It was natural to attribute these to some surface phenomena.

Recent researches by the author make it evident that in ordinary liquids aggregations and disaggregations must inevitably take place, and that, at a given temperature and concentration the liquid must contain simultaneously several molecular species, which are capable of reversible transformation into one another. The equilibrium between these molecular species is not *necessarily established instantaneously* and sometimes requires many months.

These subjects are of great interest in biological science, as evidenced by the fact that in many biological treatises one finds cited a law discovered by the author long ago, and called Antonoff's rule, or theorem (2). This law can be stated as follows:

When two liquid layers are in equilibrium with one another, the interfacial tension $\gamma_{1,2}$ is equal to the difference between the surface tension γ_1 and γ_2 of the two layers measured against their common vapor:

$$\gamma_{1,2} = \gamma_1 - \gamma_2 \quad (\text{Law 1}).$$

The statement above differs somewhat from that given by Gortner (2), and the reason is as follows.

It was formerly thought that it is sufficient to mix the two liquids thoroughly, bring them to thermal equilibrium, allow them to separate into two distinct layers, and carry out the measurements. Nowadays it is known that, in accordance with the above procedure, the experimental data are often at variance with the requirements of the Law 1. For example, in a system isoamyl alcohol-water the deviation from the above law may be as great as 400%. This phenomenon seemed obscure for several decades, and only quite recently it became clear that such deviations are due to the fact that the system is not in equilibrium.

Experience showed that the procedure mentioned above is insuffi-

cient to bring a system into equilibrium, in spite of the fact that thermal equilibrium is reached within half an hour. These phenomena can only be understood in the light of the author's theory.

II. THEORY OF SOLUTIONS

The earlier account of the theory can be found in a number of papers published in *Phil. Mag.* and other periodicals (3, 4, 5, 6, 7, 8, 9) and in its more final form in *J. Phys. Chem.* (8). According to this theory two partially soluble liquids forming two superposed solutions can coexist without mixing because they are iso-osmotic, or isotonic as it is often called.

If they are in equilibrium, these two solutions have identical colligative properties,* *i.e.*, 1) they emit the same vapor, their vapor pressure

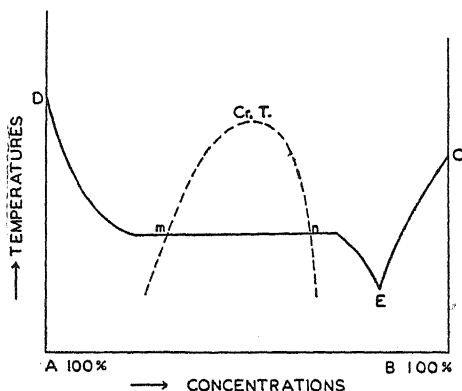


FIG. 1

and composition of vapor are identical, 2) they boil at the same temperature, 3) they freeze at the same temperature.

The freezing point curves are particularly instructive. They are generally of the type shown in Fig. 1, where the freezing temperatures are plotted along the ordinates, and concentrations along the abscissae. Let us determine the freezing point of the pure substance B. On adding

* Colligative or osmotic properties of solutions are those which depend mainly on the *number*, and not the *nature* of the molecules present. In this case, the vapor pressures, the boiling points and the freezing points, all of which are related to the osmotic pressure, are meant.

to it the substance A, one can observe that the freezing points will become lower and lower until the eutectic point E is reached. All along the curve *DE* it is the substance A which separates as ice. In this case it is convenient to regard the substance A as the solvent.

On the right hand side of the point E, between E and C, the substance B will separate as ice.

When point *n* is reached the solution ceases to be homogeneous and a second liquid layer appears. On further addition of A the relative amount of the second layer increases, and at *m* only the second layer is available. At both points *m* and *n*, the freezing temperatures are the same, and on further addition of A the curve begins to rise. Points *m* and *n* correspond to concentrations of the two superposed liquid layers in equilibrium with each other. They are both situated on the left hand side of the eutectic point and they both separate the same ice. *Therefore they must be regarded as two solutions in the same solvent.* The dotted curve, having generally the shape of a slightly asymmetric parabola, is the solubility curve. Outside this curve the system is homogeneous. Inside the curve it is heterogeneous, *i.e.*, it consists of two liquid layers.

The top of this parabola is the so-called critical point of dissolution, or the consolute point. At this point, the system separates into two layers of equal volume. Above the critical point the system is homogeneous. It is interesting to note that all around the critical point the solutions exhibit the phenomenon of opalescence. It is also remarkable that some degrees above the critical point the colligative properties continue to be independent of concentration and their curves remain horizontal well outside the solubility curve.

Thus, in accordance with the diagram of Fig. 1, to the substance A, which is the solvent, substance B is being added. When a certain concentration is reached the freezing point curve becomes horizontal. This would indicate that the molecules of substance B added to the solution combine with molecules in solution without increasing their number. On further addition there is separation into two layers.

Thus the two liquid layers in equilibrium with each other must be regarded as solutions in the same solvent, containing the same number of molecules per unit volume (Law 2).

It means that the substance dissolved has in one layer a different molecular status than in the other. Thus the equilibrium may be of

the nature

$$n B \rightleftharpoons B_n$$

and it must shift as a function of concentration, and also as a function of temperature, in a reversible way.

Formation of complex molecules starts in the less concentrated layer, as evidenced by the fact that the curve of colligative properties becomes horizontal before saturation is reached.

Thus, one can expect that in these solutions several molecular species will be present simultaneously in each layer, those in the more concentrated layer being of a higher degree of complexity.

The equilibrium may require some time to be established.

When solutions of various concentrations are used for determining the change of various physical properties as a function of concentration, the curves obtained are as a rule discontinuous. This can be due to two causes, *viz.*, the system may not be in equilibrium, in which case the curve will change its aspect with time. Or else further addition of the dissolved substance causes such changes in the system that the curve suddenly departs from the smooth path forming an angular point. The same happens when properties are studied as a function of temperature. The measurements of densities is particularly instructive because they may be carried out with extreme precision. These curves exhibit distinct signs of discontinuity. The inflection points in these curves can only be interpreted as an evidence of molecular changes within these solutions.

III. THEORY OF PURE LIQUIDS

The theory outlined above was extended and it became possible to explain some peculiarities in the behavior of pure liquids.

This theory originated in the following way. Having worked on critical phenomena, the author was impressed by the fact that a striking analogy exists between the critical phenomena of two kinds.

1) When a pure liquid is heated at a certain temperature and pressure, it reaches its critical point, above which it can only exist as a gas and no amount of pressure is capable of condensing this gas to a liquid. At the critical point, the substance separates into two phases, liquid and vapor, of equal volume. Also, at the critical point the densities of the two phases become identical. Going down the temperature scale, the densities of the liquid increase, and those of the vapor diminish.

If one plots densities against temperature one gets a curve having the shape of an asymmetric parabola. By taking half the sum of the densities of the liquid and that of the vapor, one gets a straight line (Law of Cailletet-Mathias, Fig. 2).

2) If we take a mixture of two liquids with limited solubility, such as phenol and water, we can observe a similar critical phenomenon. A critical temperature of dissolution (or a consolute point) exists above

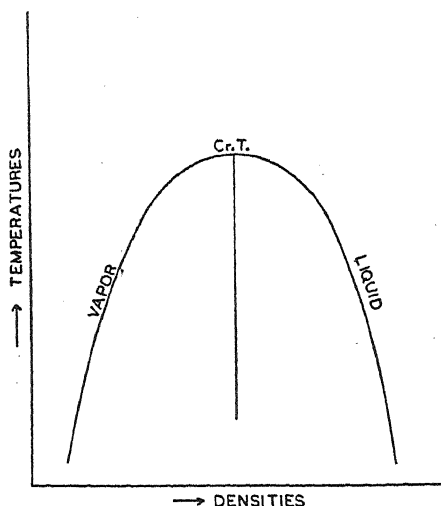


FIG. 2

which the system becomes homogeneous. Just below this point the system separates into two *liquid* layers of equal volume. The curve representing the densities of both liquid layers has the shape of a slightly asymmetric parabola, one branch of which represents the densities of a less concentrated layer, and the other representing the densities of a more concentrated layer. The law of rectilinear diameter is exemplified just as in the case mentioned above.

If one compares the two critical phenomena, one can see that they are identical in every way, except that in one case substance condenses within a space not otherwise filled, and in the other case condensation takes place within a solvent. According to the theory of van't Hoff, the dissolved substance very often behaves as if it were in a gaseous state and the solvent is only a medium which remains quite inert as if it

did not exist at all. Thus, one can conclude that the dissolved substance behaves as a vapor in the less concentrated layer and in the more concentrated layer as a liquid.

From this analogy, we can conclude that Law 2 can be extended to pure liquids with all its consequences; *i.e., vapor and liquid in equilibrium with each other must contain the same number of molecules per unit volume*. The difference in densities of vapor and liquid, small in the vicinity of the critical point, increases at lower temperatures. This shows that the liquid must necessarily be associated, and the degree of association increases as the temperature decreases.

It also follows that at any temperature one can expect the presence of more than one molecular species in liquids. The equilibrium between these may require some time to be established.

At first glance, this view appears to clash with the generally accepted theory that normal and associated liquids exist. The former are believed to have the same molecular weight in the liquid state as in the vapor state.

The evidence adduced by the author supports the view that all liquids behave in like manner, or at least their kinetic behavior is such that they must be assumed to contain complex molecules. There is, however, an essential difference between different liquids as regards the nature of the bonds holding the molecules together in a complex and in the so-called "associated liquids" such as organic acids the formation of a complex is accompanied by structural changes. This question will be discussed again at a later stage.

It is noteworthy that densities and some other properties of liquids change discontinuously as a function of temperature. This may be due to either or both of the two following causes. The liquid on reaching a certain temperature is not necessarily in a state of equilibrium, and the inflection in the curve may be due to some new molecular change within the liquid. This is shown in Fig. 3 in which the latent heats of evaporation are plotted against the temperature for two hydrocarbons showing marked discontinuity. These data have been borrowed from the classical experimental work by Sydney Young (10).

It is highly significant that the discontinuities in properties are exhibited by all liquids for which accurate data exist, irrespective of their nature. Thus, benzene or other hydrocarbons also show them. The rectilinear diameter, if one looks into the matter more closely, is a zig-zag line and not a straight line as generally assumed (7).

Also the experiments by H. B. Baker on excessive drying of liquids gave results which can only be explained in the light of this theory. Thus if benzene, for example, has quite a different vapor pressure and boiling point in an excessively dried state, it can be regarded as a system in which the several molecular species in the liquid are not in

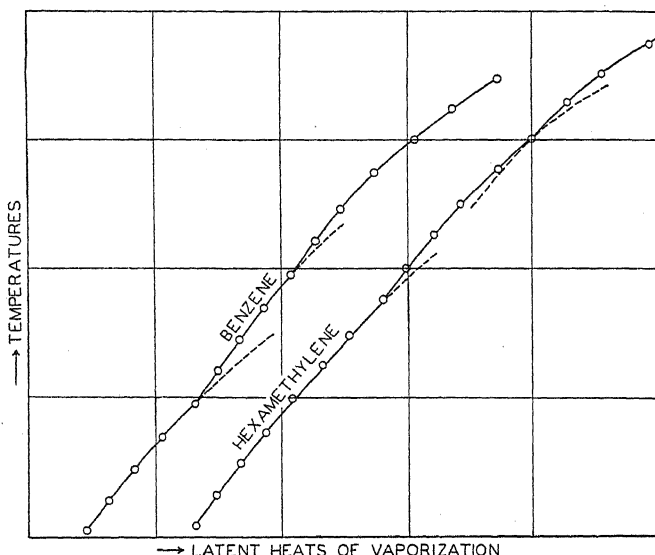


FIG. 3

equilibrium. The moisture apparently acts as a catalytic agent helping the system to attain the state of equilibrium. The attention of the author was drawn to this fact by the late H. Freundlich. The difference in the behavior of various liquids apparently is due to the fact that the time necessary to reach equilibrium varies within wide limits. Thus, in water the physical properties acquire a fixed value immediately after the thermal equilibrium is reached. This is probably because water is an ionic substance, consisting of two inorganic ions. It is well known that the ionic reactions as a rule proceed so rapidly that they appear to us to be instantaneous and their velocities cannot be measured.

IV. EXPERIMENTAL DATA

Experiments with Solutions

As a result of this theory, the author was conscious of the fact that the colossal deviations observed by Harkins from Law 1 could only be due to the fact that the systems investigated by him were not in a state of equilibrium. The work of Harkins is of great importance because he was the first to have the idea of working with highly purified liquids, and such systems show a remarkable stability in spite of the fact that they may be very far from the state of equilibrium. Harkins worked on a quite different theory, which could not give him the idea that the system had to be kept under observation for a number of months. As the system showed no tendency to change with time, Harkins concluded, and with full justification, that Law 1 was far from being true. The present author, as a result of his theory, knew that every system is bound to attain the state of equilibrium if it is allowed to stand long enough.

Thus, an experiment was made with chemically pure isoamyl alcohol and water, forming two liquid layers, as illustrated in (12). The system began to show tendency to change only after about 20 days of standing in a thermostat. Then the properties fluctuated during another 20 days and only after about 50 days were the fluctuations no longer observable, and the system in equilibrium showed constant physical properties.

The difference between the experiments of the author and Harkins is that the author showed more patience and continued the experiments over a much longer period. In carrying this experiment out the author had difficulty with his collaborators who wanted to discontinue the experiment as hopeless. Of course, the author would probably have been inclined to do so himself, if it were not for the fact that he already had experience with other systems in which equilibrium is established in 5-10 days and which did not require any special patience for their execution.

Fig. 4 represents an experiment with the system phenol-water in which, by means of surface tension measurements, the effect can be shown to be so great that nobody will ever attribute it to experimental errors (13).

The above mentioned two substances have an alcoholic function and both of them exhibit changes in properties as a function of time, but it is not known yet whether these changes are accompanied by structural changes or not. In the case of the phenol-water system, experiments with Raman spectrum have been made by Venkateswaren and Pandya which, however, could not give conclusive evidence as to existence of structural changes because of some peculiarities in the Raman spectrum of this system (11).

It was thought that an experiment with a substance with acidic function might be of interest. Isobutyric acid was chosen for this purpose, because in mixture with

water it gives a critical mixture separating into two liquid layers with the critical temperature at about 29°C .

The system was first frozen and then the two layers kept at 11.5°C . in a thermostat.

The change in properties is shown in (12).

Apart from this an 8% solution of isobutyric acid was prepared and its density measured during 40 days. It showed a distinct increase in density from the moment of preparation of the solution.

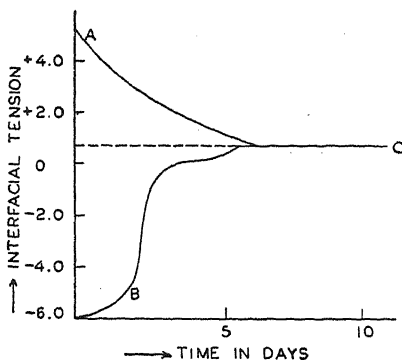


FIG. 4

Experimentation with the Raman spectrum gives evidence that there is a definite structural change resulting in a formation of double molecules as one approaches the critical temperature from above. This is highly significant from the point of view of the author's theory, according to which in the critical region the equilibrium is characterized as being due to the presence of an equal number of single and double molecules (5, 6).

In these experiments we can see the evidence of molecular changes, but it can be said that the presence of water in the solutions in question may have something to do with it.

For this reason another set of experiments was performed in which water was excluded as much as possible, without excessive drying of the sample or ultra drying as the technique of H. B. Baker is sometimes called.

For the first substance normal propyl alcohol was taken. It was dried by the ordinary methods and distilled. Immediately after that a curve of densities as a function of temperature between 20 and 80°C . was determined in twin pycnometers. They were kept at a given temperature for 20 to 30 minutes, the time necessary and sufficient for attaining thermal equilibrium. The twin pycnometers always agreed within .0001, which is quite satisfactory for the purpose of this research.

The liquid was left in a basement with a fairly constant temperature.

After a period exceeding 6 months the same liquid gave very different results. It thus appears that distillation has disturbed the equilibrium and during subsequent months changes were taking place leading to a state of equilibrium (9). As the alcohols

do not freeze it was not possible to see whether these changes affect the melting point of the substance.

The substance proved unsuitable for study of absorption spectra and it is therefore difficult to say whether these transformations are accompanied by structural changes or not.

As a second example of a pure substance phenol was chosen. A c.p. phenol was taken for these experiments and distilled. Phenol is liable to undergo some irreversible changes recognizable by the red color resulting from oxidation by air in the presence of ultraviolet light. The presence of moisture appears to be necessary for this reaction.

The pure phenol thus prepared was kept in the dark in a glass vessel closed by means of a thick piece of rubber. After many months it remained quite colorless, yet its melting point was very much lower than originally.

The pure phenol after distillation is not hygroscopic; therefore the lowering of the melting point after standing can only be explained as a result of some transformation the nature of which is not yet understood in view of failure of the experiment with Raman spectrum. This is apparently correlated with transformations taking place in solutions of phenol in water which are similar in their effects to those of isobutyric acid solutions. There are various possibilities, one of them being the transformation of phenol into a keto form, long suspected but never actually established. However, the author firmly believes that in the phenol-water system the change at the critical point must be accompanied by formation of double molecules, as is the case with isobutyric acid solution, which he emphatically pointed out some years ago (5, 6).

Thus, one and the same chemically pure substance can have substantially different properties according to the relative amounts of different molecular species in the system. While the system approaches the state of equilibrium, one can *observe enormous fluctuations in the surface tensions of both layers and also in the interfacial tension*. But, what is most striking, *the densities of both layers fluctuate so much that one observes differences in the third decimal place* (sometimes even in the second place) (12). It should be emphasized that the density measurements can be easily made to six decimal places.

When the system reaches equilibrium, all these fluctuations cease to manifest themselves and it is only then that one can speak of any definite physical properties of these liquids. The fact that there are such enormous density changes is the best proof *that it is not a surface phenomenon*.

These chemical processes are essentially reversible, although there are cases where irreversible processes such as oxidation, etc., also take place. If the system was previously kept in the cold and then placed in a constant temperature bath, thermal equilibrium is attained in about 15 or 20 minutes whereas chemical equilibrium may take weeks or even months.

If the substance was kept at high temperatures, on being placed in the bath, it will exhibit different properties, as in the case above, but the end product will be the same in both cases. This can be demonstrated easily on the system phenol-water (13).

The curves leading to equilibrium are not necessarily reproducible. Taking samples from the same bottle, one generally gets fairly good reproducibility, but another sample may give different results because the previous history and impurities may not be the same. However, every system can be brought into equilibrium in conformity with Law 1 unless there are irreversible changes.

Similar phenomena to those observed in liquids and solutions have been described by different authors in colloidal systems.

In various publications of Nord (14) and his collaborators one can see a definite allusion to the fact that the properties of colloidal systems often depend on their previous history, the effect of previous freezing being particularly apparent. According to him the effects of freezing generally result in aggregation or disaggregation, which may be reversible or irreversible, as the case may be. These changes may or may not be accompanied by structural alterations. From the point of view of the author the case of sodium oleate solutions, where Nord observed a marked change in volume as a result of previous freezing is very interesting. Here we have an organic ion with colloidal properties behaving analogously to the isobutyric acid described by the present author.

Further allusion to these facts is found in a paper on soaps and fats by Thiessen (15). In such colloids, according to him, equilibria exist depending on temperature and concentration, which follow the changes of external conditions only very slowly.

The same applies to casein where the process of aggregation or disaggregation appears to be reversible. Similar phenomena were observed by Sorensen (16). In the case of polyacrylic ester in alcohol, cooling produces first coagulation and after this the process is reversible.

Spectra of solutions of polyvinyl alcohol previously frozen undergo change which depends on change in diameter of particles. Without any structural changes (14) a 0.5% solution of polyacrylic acid in 2% NaOH which was not subjected to cold, spectroscopically was identical with a more concentrated solution which was frozen 24 hours before and diluted to 0.05%.

G. Scheibe and collaborators (17) observed reversible polymeriza-

tion in dye-colloids with changes of temperature. There were conductivity changes observed with appearance of new adsorption bands.

This is different from solutions of polyacrylic acid, as observed by Nord (14) where no conductivity changes and no new bands were found. Thus, aggregation-disaggregation is not necessarily a simple process of polymerization but, in some cases, something else is involved in it.

The Same Phenomena in Biological Problems

The facts related in previous paragraphs are of interest in a number of biological problems. The reversible changes observed by the author with isobutyric acid can explain the interesting results recorded by B. Lustig and H. Wachtel (18) who found that some organic acids have essentially different physiological effects if they were previously boiled. However, when allowed to stand a number of days or weeks, again they acquire properties similar to those exhibited when they have not been boiled at all.

In the problem of activity of viruses similar phenomena make themselves felt. For example, the Tobacco mosaic virus solution separates on standing into two layers (19). It is not stated, however, whether this process is reversible. But the very fact that it requires some time is of interest, because in most known cases this time is very small, or even negligible.

There exists also evidence indicating that Foot and Mouth disease virus proteins in solutions reach a state of equilibrium, and that the infectious activity of a virus solution reversibly changes with temperature, although the state of equilibrium is by no means instantaneous, and sometimes needs a considerable time.

These are not, however, the only facts of this kind known. In the literature one occasionally finds allusion made to them with insufficient emphasis to attract attention.

Thus, for example, some authors (20) attribute the effects of freezing of plants to the changes taking place in the colloidal matter of the protoplasm, and particularly to irreversible processes of the type described by Nord.

CONCLUSIONS

1. The so-called physical constants of liquids frequently vary within wide limits. Most experimentalists attribute these differences to experimental errors.

2. Surface tension measurements served better than the density determinations since the former yield values which cannot be attributed to experimental error. The density variations which commonly affect the third decimal place are also well outside the limits of experimental errors, considering that densities can be determined accurately to the sixth place, but they impress the readers much less.

3. In the development of these subjects, of course, the theory as outlined above is of paramount importance, because it made it obvious that properties of liquids must depend on their previous history. It thus became possible to plan the research in such a way that the time factor was introduced as a definite parameter. On the other hand, there are quite a number of isolated researches which have not attracted due attention, and which in the light of this theory acquire great importance, indicating that these phenomena are of a much more general character than first appeared. Amongst these facts we mentioned the results recorded by F. F. Nord and collaborators on cryolysis and allied subjects (1).

4. This theory opens new vistas in the field of biological science, as illustrated by the work of Lustig and Wachtel on the physiological effects of liquids as a result of their previous history, and that of Pyl on activity of the viruses, the effect in this latter case being subject to reversible changes which take some time for their equilibration.

SUMMARY

In the course of surface tension measurements, enormous discrepancies are observed in properties of liquid systems separating into two liquid layers. The properties of pure liquids may also vary within wide limits. The explanation is as follows:

There exists a close analogy between systems separating into two liquid layers and pure substances which condense below their critical point. In the first case, a liquid condenses within a solvent and forms two liquid layers. In the second, the solvent is absent and an individual substance condenses in vacuo. In the first case the two phases contain an equal number of molecules per unit volume because they are iso-osmotic. Thus the system must contain more than one molecular species in solution. The pure substances below their critical point behave likewise.

The different molecular species are not necessarily in equilibrium

because the process of equilibration may be slow. These changes are in most cases reversible but they sometimes respond to changes of temperature and concentration very slowly. Thus, the time factor is an essential parameter in the study of liquids, which acquire definite properties only if they are brought into a state of equilibrium. Hence, properties of liquids must depend on their previous history.

This paper emphasizes the importance of these phenomena in the realm of colloids and in biological problems, of which several examples are given.

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The Effect of Nicotinic Acid and Nicotinamide on Growth, Liver Fat, Renal Hemorrhages and Excretion of "Trigonelline" in the Rat ¹

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INTRODUCTION

Ackermann (1) recognized the presence of trigonelline, a methylated derivative of nicotinic acid, in the urine of dogs which had received a dose of nicotinic acid. Only recently have other investigators, Handler and Dann (2), Stekol (3) and Challenger (4), become interested in the origin of the methyl groups necessary for the synthesis of trigonelline. If these methyl groups derive from the same pool from which other methylated compounds draw, excessive amounts of nicotinic acid would deprive the organism of methyl groups and produce or aggravate the symptoms of methyl group deficiency. The work of Borsook and Dubnoff (5) and du Vigneaud (6) suggested that a similar deprivation of methyl groups may occur when creatine is formed following the administration of guanidoacetic acid. This was proved by the work of Stetten and Grail (7). Du Vigneaud (6), Best (8) and Griffith (9, 10) have described the syndrome of methyl group deficiency. It manifests itself by fatty infiltration of the liver, renal hemorrhages, and inhibition of growth. These symptoms can be prevented or cured by the administration of "methyl donors," such as methionine or choline. Incidental to other studies, Forbes (11) found

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that nicotinic acid and nicotinamide increase the fatty infiltration of the liver, and Griffith and Mulford (12) found that choline did not prevent hemorrhagic degeneration of the kidneys as effectively when the diets contained nicotinic acid.

Stekol (3) published a preliminary report in which he stated that nicotinic acid has an inhibitory action on the growth of male rats which is not prevented by choline but is prevented by methionine. The latter substance was believed to supply the methyl groups necessary to prevent the deficiency created by an excessive production and excretion of trigonelline. This hypothesis was strengthened by the observation that in the female rat, which does not excrete trigonelline, nicotinic acid does not inhibit growth. Challenger (4) suggested the possibility that the donor of methyl groups for this biological synthesis might be choline as well.

The purpose of this work was to investigate the effect of nicotinic acid and nicotinamide, with and without added choline, on the growth of young rats, the fatty infiltration of the liver, the hemorrhagic degeneration of the kidneys, and the urinary excretion of trigonelline.

EXPERIMENTAL

The experimental animals were male rats of the Wistar strain. In preliminary experiments, 179 animals from 3 to 5 weeks old and weighing from 19 to 115 g. were divided into groups and allowed to eat at will, each group in a separate cage. The basal diet consisted of lard 20, Mazola oil 20, cod liver oil 3, salt mixture 4 (Osborne and Mendel, 13), with variable amounts of casein (Labco, vitamin-free), nicotinic acid, nicotinamide, as indicated in Tables I and II, and sucrose to make 100, plus 1 tablet (0.5 g.) of brewer's yeast. Choline was added in amounts containing theoretically enough methyl groups to transform all nicotinic acid or nicotinamide present into trigonelline or a multiple of that amount.³ When the effect of different dietary supplements was to be compared, littermates of uniform weight were used. The food was weighed daily.

The rats which received diets with 0.5 per cent nicotinic acid had a higher average liver fat than the animals on the basal diet. The average liver fat of the animals receiving diets with 3 per cent nicotinic acid plus 1 per cent choline or 1 per cent nicotinic acid and 0.17 per cent choline was much lower than in rats receiving the control diet. The differences were more marked in the rats fed a 5 per cent casein diet than in those who received diets containing 10, 18, or 30 per cent casein diets.

The liver fat was determined also in 77 adult rats varying in weight from 222 to 306 g. and receiving similar diets. Neither nicotinic acid nor nicotinamide increased

³ The difference of 1 in the molecular weights of nicotinic acid and nicotinamide was disregarded.

the liver fat above the basal level; choline, however, reduced it to lower values. The adult rat is stated by Griffith and Wade (14) to be less susceptible to fatty infiltration of the liver.

Renal hemorrhages were more severe in the rats eating diets supplemented with nicotinic acid than in the control animals. The lesions were prevented by choline. No hemorrhages were observed in the kidneys of adult animals.

TABLE I

Effect of Nicotinic Acid, Nicotinamide, and Choline on Food Intake and Growth of Pair-Fed Rats

Growth curve	No. of pairs	Average weight at start	Diet				Daily food intake	Daily gain in weight
			Casein	Nicotinic acid	Nicotinamide	Choline		
		<i>g.</i>	<i>per cent</i>				<i>g.</i>	<i>g.</i>
A	6	58	5	—	—	—	2.7	0.1
B		59	5	—	—	2	2.6	0.1
C	6	51	5	—	—	2	2.6	0.1
D		52	5	3	—	2	2.6	0.1
E	4	51	18	—	—	—	2.7	0.8
F		53	18	—	0.5	—	2.7	0.7
G	6	51	18	—	0.5	—	3.0	0.7
H		51	18	0.5 ✓	—	—	3.0	0.7
I	6	43	18	—	0.5	—	3.7	1.4
J		41	18	—	0.5	0.17	3.7	1.5
K	6	57	18	—	—	—	4.5	2.1
L		55	18	0.5	—	0.17	4.5	2.2
M	6	50	18	0.5 ✓	—	—	4.7	2.4
N		51	18	—	—	—	4.7	2.8

The rate of growth was slower when nicotinic acid or nicotinamide was added to the basal diet. However, the animals receiving these additions consumed much less of their diet. This effect was not prevented by the further addition of choline to the diet.

Because of this, and because of a wide variation in the concentration of fat in livers of rats of the same group with considerable overlapping of the values for liver fat between groups, it was considered necessary to investigate the problem further. For this purpose, one hundred and two young rats were kept in individual cages

TABLE II

Effect of Nicotinic Acid, Nicotinamide, and Choline on Liver Fat and Renal Hemorrhages in Pair-Fed Rats

Series number	Number of pairs	Duration of experiment	Daily food intake	Diet				Average liver fat	Ave. weight Body weight		Kidney hemorrhages
				Ca-sein	Nico-tinic acid	Nico-tinamide	Choline		Liver	Kidneys	
		<i>days</i>	<i>g.</i>					<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
1	7	29	4.5	18	—	—	—	35.2	6.3	1.1	+
2				18	0.5	—	—	39.8	7.1	1.3	++
3	6	25	2.7	18	—	—	—	22.2	6.4	1.3	+
4				18	—	0.5	—	27.1	8.3	1.7	+++
5	6	30	3.0	18	0.5	—	—	28.2	6.4	1.2	+++
6				18	—	0.5	—	28.1	6.4	1.2	+++
7	8	30	4.3	18	—	—	—	24.4	6.7	1.1	+
8				18	0.5	—	0.17	9.4	4.1	0.9	None
9	4	30	4.9	18	0.5	—	—	39.4	6.8	1.1	++
10				18	0.5	—	0.17	12.4	3.8	1.1	None
11	6	18	3.7	18	—	0.5	—	38.1	6.9	1.3	++
12				18	—	0.5	0.17	11.2	3.6	0.9	None
13	2	30	3.5	18	—	—	—	27.3	6.1	1.1	?
14				18	—	—	0.17	10.2	3.1	0.9	None
15	6	24	2.3	5	—	—	2	8.5	3.9	1.0	None
16				5	3	—	2	9.0	3.9	1.0	None
17	6	25	2.7	5	—	—	—	26.3	5.2	1.0	+
18				5	—	—	2	10.4	3.9	1.0	None

and pair-fed. The animals of each pair were equal in size (within 2 g.) and littermates. The food was weighed daily to the nearest gram. The basal diet had the composition above described.

Effect of Nicotinic Acid, Nicotinamide, and Choline on the Growth of Young Rats. Forty pairs of rats were used in these experiments, which lasted from 15 to 40 days. The animals were weighed daily, and the

average change in weight of each animal for a five day period was computed. The results (Fig. 1 and Table I) indicated that the rate of growth is proportional to the food intake and that no significant differ-

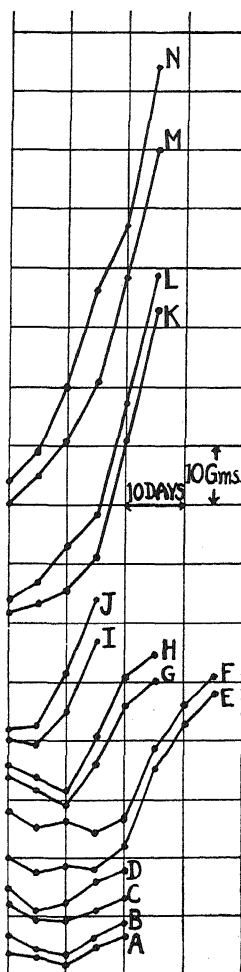


FIG. 1

Effect of Nicotinic Acid, Nicotinamide and Choline on the Growth of Pair-Fed Rats

The letters on the curves correspond to the pairs indicated in Table I.

ence exists between the animals receiving the basal diet and their pair-mates, receiving supplements of nicotinic acid, nicotinamide, or choline except possibly groups M and H. The experiments further demonstrate that there is no difference between the effect of nicotinic acid and that of nicotinamide (Curves G and H), and that the addition of choline to diets containing 5 or 18 per cent casein does not stimulate the rate of growth.

The relatively poor rate of growth of the control animals was probably due to the limitation in the food intake imposed by their pair-mate. Control animals fed the basal diet *ad libitum* consumed 8.6 g. per day and gained 3.8 g. showing that the diet was adequate. The food intake of the pair-mate also explains the difference between groups E and K (Table I) which received the same diet.

Effect of Nicotinic Acid, Nicotinamide, and Choline on the Fatty Infiltration of the Liver. Fifty-one pairs of growing rats were used; these include the 40 pairs used in the growth experiments. The animals were killed by stunning, decapitated immediately, and bled. The livers were examined grossly and weighed. The liver fat was determined by the method described by Tucker and Eckstein (15). The results (Table II) indicate that the addition of 0.5 per cent nicotinic acid or nicotinamide to an 18 per cent casein diet produces an increase in the weight and the lipid content of the liver, and that no difference exists between the action of these two substances; this action is prevented by choline. It will be observed that the rats fed the basal ration had a high liver fat. This is probably due to the high fat content of the diet (40%) and, in the case of groups 15-18, to the low casein content (5%).

Effect of Nicotinic Acid, Nicotinamide, and Choline on the Hemorrhagic Degeneration of the Kidneys. The kidneys, obtained from the same animals mentioned in the preceding paragraphs, were inspected for hemorrhagic degeneration and weighed. The weight of the kidneys has been reported by Griffith and Wade (16) to be proportional to the severity of the hemorrhagic disease, and this proportionality was confirmed in our experiments (Table II). The results indicate that rats fed supplements of nicotinic acid or nicotinamide show an increase in renal weight and hemorrhages, which is prevented by the administration of choline. The hemorrhages were more severe in rats fed an 18 per cent casein diet than in those fed a 5 per cent casein diet. This fact is in agreement with the findings of Griffith and Wade (14, 16) that no hemorrhagic degeneration occurs unless the diet contains sufficient

protein to permit growth. Again no difference was found between the action of nicotinic acid and nicotinamide.

Effect of Nicotinic Acid, Nicotinamide and Choline on the Urinary Excretion of "Trigonelline." Six adult rats (275–355 g.) were placed in individual glass metabolism cages with double screen bottoms to prevent coprophagy. Care was taken to prevent food scattering. The urine was collected into bottles containing 5 cc. of 3.5 *N* sulfuric acid and a few drops of toluene. Once a week the cages were washed with distilled water and the washings added to the urine for the determination of nicotinic acid and trigonelline. The animals were fed the basal diet containing 5 per cent casein. Supplements of nicotinic acid, nicotinamide, and choline were added as indicated in Table III with a

TABLE III

Effect of Choline on the Urinary Excretion of "Trigonelline" in Rats Fed Supplements of Nicotinic Acid or Nicotinamide

Supplements	Duration	Nicotinic acid excretion	Trigonelline excretion	Trigonelline Total nicotinic acid	Nicotinic acid intake	Nicotinic acid recovery
	<i>days</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>
None	25	0.22	0.14	38.9	—	—
0.5 per cent nicotinic acid	23	33.2	1.5	4.3	43	80.6
0.5 per cent nicotinic acid + 0.5 per cent choline	27	42.4	3.8	8.2	46	101.2
0.5 per cent nicotinamide	21	20.0	3.0	13.0	34	67.7
0.5 per cent nicotinamide + 0.5 per cent choline	22	12.9	4.3	25.0	37	46.5

The figures represent the average results obtained in 6 rats. All values are expressed in milligrams of nicotinic acid per rat per 24 hours. One rat died after 10 days on the diet containing 0.5 per cent nicotinic acid plus 0.5 per cent choline; 2 rats died after 10 days on the diet containing 0.5 per cent nicotinamide and 0.5 per cent choline.

corresponding reduction in the amount of sucrose. Each supplement was fed for a period of 21–27 days with the exceptions noted in Table III. Nicotinic acid was determined by the method of Melnick and Field (17) and trigonelline by the method of Fox, McNeil, and Field (18). Average figures are reported as the results were qualitatively the same in each of the six rats used.

The results indicate that about 40 per cent of the total nicotinic

acid excreted is in the form of trigonelline, when the rats are on basal rations. When nicotinic acid or nicotinamide are added to the diet, more trigonelline is excreted, the increase being greater with addition of nicotinamide. However, the percentage of total nicotinic acid excreted as trigonelline in the urine drops considerably. This fact has been reported by Handler and Dann (2). When choline was added to a diet containing either nicotinic acid or nicotinamide, the excretion of trigonelline increased significantly. It appears, therefore, that the rat can utilize the methyl groups of choline for the production of trigonelline from nicotinic acid and nicotinamide and that more trigonelline is produced from nicotinamide than from nicotinic acid.

DISCUSSION

While this work was in progress, Handler and Dann (2) published the results of a similar study. These authors state that nicotinamide inhibits the growth of rats, that nicotinic acid has only a slight effect upon growth and that choline does not prevent this action unless homocystine is present. In our experiments, when the food intake was controlled there was no difference in the rate of growth of animals receiving the basal ration and of those receiving supplements of nicotinic acid, nicotinamide, or choline. It would seem probable, therefore, that the differences in the rate of growth of Handler's and Dann's rats was not due to a specific growth inhibiting effect of nicotinamide but might be entirely accounted for by differences in food intake of the various groups of animals.

The existence of a relative methyl group deficiency produced by excessive amounts of nicotinic acid or nicotinamide, which was not revealed by a specific effect on the growth of young rats in our experiments, was demonstrated by the increase in liver fat. Nicotinic acid and nicotinamide were found to be equally effective in producing fatty infiltration of the liver, when fed in equal amounts, contrary to the finding of Handler and Dann that nicotinic acid has a greater effect. In the experiments of these authors, nicotinic acid produced more fatty infiltration of the liver than nicotinamide, probably because the former has a smaller inhibitory effect on food intake and growth. It is believed that, also in this case, the results of Handler and Dann can be explained by the difference in food intake between the animals receiving supplements of nicotinic acid and those receiving supplements of

nicotinamide, rather than by a difference in the mechanism of action of the two substances. Recently Handler (19) has demonstrated that the rabbit and guinea pig do not methylate nicotinamide and that this substance has no effect on growth and liver fat and that it does not have other noticeable toxic effects on these animals. The author concludes that this evidence supports the previous conclusion of Handler and Dann that the "toxicity" of nicotinamide is due only to the deprivation of methyl groups. We agree with this statement and believe that it can be applied equally well to nicotinic acid. The similarity of action of the two substances becomes apparent when the food intake is controlled.

The addition of nicotinic acid and nicotinamide to the diet of growing rats produces more severe renal hemorrhages than does the basal diet alone. The similarity in the action of these two substances in producing a methyl group deficiency is again apparent.

The study of the urinary excretion of nicotinic acid and its derivatives revealed that both nicotinic acid and nicotinamide can utilize the methyl groups of choline for the formation of trigonelline. It is possible that the methylated derivative determined by us in the urine of the rat might be, at least in part, *N*¹-methyl-nicotinamide, recently discovered by Huff and Perlzweig (20). However, we have preferred to use the term "trigonelline" as our analytical procedures does not differentiate between these two substances.

SUMMARY

1. Nicotinic acid, nicotinamide, and choline have no specific action on the growth of young rats. Nicotinic acid and nicotinamide inhibit the growth of the animal only because food intake is inhibited by these supplements. Choline did not prevent growth impairment from nicotinic acid or nicotinamide, when the food intake was controlled. When the food intake is controlled no difference exists between the rate of growth of animals receiving nicotinic acid, nicotinamide, or choline, and that of their pair-mates.

2. Nicotinic acid and nicotinamide equally increases the fatty infiltration of the liver and the severity of the hemorrhagic degeneration of the kidneys. This action is prevented by choline.

3. Rats excrete more trigonelline when they receive supplements of nicotinamide than when nicotinic acid is fed. Choline added to a diet

containing nicotinic acid or nicotinamide produces a further increase in the urinary excretion of trigonelline.

4. It is believed that nicotinic acid and nicotinamide obtain methyl groups from choline for the production of methylated derivatives.

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Observations upon the Creatine Content of the Muscles of Normal, Biotin Injected and Biotin-Deficient Rats

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INTRODUCTION

In a recent paper Nielsen and Elvehjem reported the results of feeding rats on their biotin-deficient diet (1). The statement was made that the concentration of muscle creatine in the biotin-deficient animals was abnormally high and that administration of biotin decreased the muscle creatine. In 8 of their animals suffering from spastic paralysis the muscle creatine varied from 0.43 to 0.46 per cent, average 0.43 per cent. In another 5 controls, in which the symptoms of biotin deficiency were either prevented or cured by the administration of biotin, the muscle creatine varied from 0.32 to 0.41 per cent, with an average of 0.37 per cent, or a decrease of about 15 per cent as compared to the muscle creatine in the biotin-deficient animals discussed above.

In animals suffering from paralysis due to the ingestion of vitamin-E-free diets, or in many cases of the myopathies, the creatine content of the muscles is below normal and creatinuria usually occurs (2, 3). It was, therefore, of interest to us to determine the effect of a biotin deficiency in rats as compared to their controls fed on Purina Dog Checkers and also to observe the effect of injection of biotin on the concentration of muscle creatine as compared to the muscle creatine in normal animals without the biotin injections.

EXPERIMENTAL

Our basal diet was identical with that used by Nielsen and Elvehjem (1) except that we used Harris' vitamin-free casein and Osborne and Mendel salt mixture. We also used their technique throughout. Our rats weighed from 128 to 187 g. at the beginning of the study and had been raised on the checkers since weaning. Eighteen

rats were used, 9 fed on the basal diet of Nielsen and Elvehjem and 9 on the checkers. The biotin content of the checkers is evidently adequate for the normal nutrition of the rat, as we have produced many animals weighing as much as 400 g. on this diet. The study lasted 136 days, at which time all animals were killed by stunning, and muscle tissue was obtained as usual from the right hind leg within 10 minutes after death. The muscle creatine was determined by the method of Rose, Helmer and Chanutin (4), using the Fisher electrophotometer for the measurement of color intensities. Samples of muscle tissue were then placed in 10 per cent formalin and in Helly's fluid for histological study.

RESULTS AND DISCUSSION

For the first two months of the study the bottoms of the cages consisted of small squares which might have caused some retention of feces which, if eaten by the animals, would probably tend to increase their biotin intake. During the remainder of the study, however, bottoms with larger squares were employed and no feces were retained. On the other hand some of the animals, since they were older than those used by the Wisconsin investigators, may have stored up sufficient biotin from the checkers before the study began to prevent a severe biotin deficiency. Regardless of the explanation it is seen from Table I that, by gross examination, rats 1 to 4 were practically normal in weight and appearance as compared to their controls, with a slight drop, however, in their creatine content. Rats 5, 8, 9 were observed to be suffering from a more severe biotin deficiency and all suffered a loss in body weight (except No. 5) and in muscle creatine as compared to the controls. In Nos. 6 and 7 extreme biotin deficiency was observed, similar to that described by Nielsen and Elvehjem (1). These two animals had the lowest muscle creatine and greatest loss in body weight of all the experimental animals. The muscles of the controls were normal on gross examination, with an average creatine content of 0.44 per cent.

Histological examination of the muscles of 8 rats, 3 normal and 5 on the biotin-deficient diets, was made. The results in Table I show only a slight atrophy of the muscles of Nos. 6 and 7 suffering from the most severe biotin deficiency, while the muscles of all the others, controls and experimentals, were histologically normal. This shows that in most cases a "biochemical lesion" (in this case a decrease in muscle creatine) occurs before definite histological evidence of the deficiency is observed.

Nielsen and Elvehjem also stated that administration of biotin caused a decrease in muscle creatine. To study this point, 10 young rats were fed on the checkers and another 10, comparable to these,

TABLE I

Comparison of the Creatine Content of the Muscles of Biotin-Deficient Rats with Their Controls Fed on Checkers

(Period of study was 136 days.)

Rat No.	Body weight			Degree of biotin deficiency		Muscle creatine	
	Initial	Final	Gain or loss	Gross	Histological	Biotin-deficient	Checkers
	<i>g.</i>	<i>g.</i>	<i>g.</i>			<i>per cent</i>	<i>per cent</i>
1	149	186	+37	0 to +	Normal	0.36	
2	140	180	+40	0 to +		0.36	
3	128	159	+31	0 to +	Normal	0.32	
4	146	193	+47	0 to +		0.38	
5	140	160	+20	++		0.31	
6	156	118	-38	+++	Slight atrophy	0.27	
7	143	102	-41	+++	Slight atrophy	0.24	
8	153	138	-15	++		0.32	
9	168	153	-15	++	Normal	0.35	
						Av. 0.32	
Checkers							
10	149	200	+51	Normal			0.40
11	171	206	+35	Normal	Normal		0.44
12	144	189	+45	Normal			0.47
13	180	235	+55	Normal	Normal		0.44
14	173	218	+45	Normal			0.42
15	163	196	+33	Normal	Normal		0.43
16	147	188	+41	Normal			0.44
17	158	211	+53	Normal			0.45
18	187	230	+43	Normal			0.45
						Av. 0.44	

were injected with 2.5 γ of biotin (free acid) daily for 3 days. Two days later all 20 animals were killed by stunning and the muscle creatine determined. The results show that the average creatine content of the muscles of the biotin injected animals was identical with that of the controls (Table II).

The difference in our results as compared to those of Nielsen and Elvehjem is due to the employment of a different type of *control animal* in our study. In their study a biotin deficiency was produced which, in their controls, was either prevented or cured by biotin administration. In ours we used checkers for the diet of our controls. Using this diet (as well as a diet containing $\frac{2}{3}$ whole wheat flour, $\frac{1}{3}$ whole milk powder, together with 1 per cent of the weight of the wheat, each, as

NaCl and CaCO_3), we have observed an average muscle creatine of 0.42 per cent in over 500 young rats in our past studies (3). Our controls in the present study averaged 0.44 per cent muscle creatine. If one averages the reported values of Nielsen and Elvehjem for their 14 animals, experimentals and controls, an average muscle creatine of 0.41 per cent is obtained, which corresponds almost exactly to the creatine content of our controls discussed above.

TABLE II
*Effect of Injection of Biotin (Free Acid) upon Concentration of
Muscle Creatine in the Rat*
(2.5 γ Biotin daily for 3 days.)

Rat No.	Muscle creatine (control) per cent	Body weight g.	Rat No.	Muscle creatine (Biotin) per cent	Body weight g.
1	0.42	76	11	0.45	87
2	0.43	84	12	0.42	91
3	0.45	72	13	0.41	78
4	0.46	88	14	0.44	88
5	0.44	91	15	0.46	93
6	0.43	75	16	0.43	102
7	0.44	83	17	0.41	88
8	0.42	92	18	0.42	77
9	0.45	88	19	0.44	87
10	0.44	95	20	0.45	93
Average 0.44		84	Average 0.43		88

The controls of Nielsen and Elvehjem had 15 per cent less muscle creatine than their paralytic biotin-deficient animals. On the other hand, we observed a decrease of 27 per cent in the creatine content of our biotin-deficient animals as compared to their controls fed on the checkers. The loss of muscle creatine and body weight was, in general, proportional to the biotin deficiency. It is of further interest that none of our experimental animals ever reached the paralytic stage. It would seem, therefore, that a partial biotin deficiency in rats causes a loss of muscle creatine, as compared to their normal controls fed on our standard stock diet of Purina Dog Checkers, and a loss of body weight similar to that observed in rats fed on vitamin-E-free diets (2, 3). Our results also indicate that biotin is necessary to maintain a normal creatine content of the muscles.

The water content of our rat muscles was not determined. From many studies with normal rat muscle, however, we can give the figure of 25 per cent total solids which is very constant (5). In the animals that lost weight during partial biotin deficiency it is evident that there was no increase in the water content of these muscles since the creatine content was below normal, and we have also shown that a sudden increase in water content of the tissues will result in creatinuria (3).

Since this manuscript was written, Lazere, Thomson, and Hines (6) have also studied this question. In two groups of rats suffering from partial biotin deficiency there was no change in muscle creatine as compared to that of the controls, which confirms our results. On the other hand, in terminal biotin deficiency the animals had a muscle creatine of 0.51 per cent as compared to 0.45 for the controls, thus confirming the results of Nielsen and Elvehjem (1).

SUMMARY

Nine rats were fed on the biotin-deficient diet described by Nielsen and Elvehjem (1) and 9 controls were fed on Purina Dog Checkers for 136 days. A decrease of 27 per cent in muscle creatine occurred in the biotin-deficient animals as compared to the muscle creatine content of their controls. This loss of muscle creatine and body weight was, in general, proportional to the severity of the biotin deficiency, showing that the *stage* of this deficiency is important in relation to the muscle creatine content. This vitamin is necessary to maintain the normal creatine level in the muscles. Only 2 out of 9 rats developed a slight atrophy of the muscles after being fed on the biotin-deficient diet. The injection of 2.5 γ of biotin (free acid) per day for 3 days did not change the creatine content of the muscles of normal young rats as compared to that of similar animals not receiving the biotin injections.

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Some Observations on the Thermal Fractionation of Gluten Protein from Acetic Acid-Ethyl Alcohol Dispersions

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INTRODUCTION

Dumas and Cahours (1843) accomplished the first thermal fractionation of gluten protein by cooling an alcoholic flour extract. Haugaard and Johnson (1930) fractionated gliadin from a 60 per cent ethyl alcohol dispersion by progressively lowering the temperature. Blish and Sandstedt (1933) discussed the separation of wheat gluten into three distinct proteins by successively decreasing the temperature of acetic acid-ethyl alcohol dispersions containing a trace of electrolyte. The three groups were designated as the glutenin group, the mesonin (or intermediate) group, and the gliadin group. When K_2SO_4 was used as the electrolyte the glutenin group was precipitated when the temperature of the dispersion was lowered to 20°–18°C. The mesonin was obtained at approximately 10°–8°C and gliadin at 0° to –4°C. Moist crude gluten was employed rather than flour, since it was found that it was almost impossible to disperse the glutenin when flour was used as the initial material. The salt concentration used in these investigations was not quantitatively specified.

Stockelbach and Bailey (1938) separated the amino acids from glutenin and mesonin in an effort to differentiate between them. The method of Brazier (1930) was followed in which the copper salts of the amino acids were obtained, and these were then separated through the solubility differences of their metallic derivatives. As definite differences in nitrogen distribution were found between the two protein fractions, Stockelbach and Bailey (1938) concluded that the two frac-

* Published with the approval of the Director of the Experiment Station.

tions are probably individual substances. Harris and Bailey (1937) studied the possibility that the relative proportions of glutenin, mesonin, and gliadin present in gluten might be related to baking strength, as registered by loaf volume. It was found that the quantity of gliadin obtained was significantly correlated with loaf volume. However, no further information relative to loaf volume was secured by the thermal fractionation of the gluten proteins in addition to that obtained from a knowledge of crude protein content.

The present study was undertaken to secure information on the effects of K_2SO_4 concentration and fractionation temperature upon the proportions of gluten protein removed from acetic acid-ethyl alcohol dispersions. As a secondary objective the effect of various methods of gluten dispersion, of wheat variety, and of papain treatment was investigated.

EXPERIMENTAL

Materials

Three hard red spring wheat flours and one durum wheat flour were used in this investigation. The hard red spring flours were commercially milled, while the durum flour was produced on an experimental unit from a composite wheat sample. One of the hard red spring flours was

TABLE I
Protein, Ash, and Moisture Contents of the Four Flours

Flour description	Protein content* ($N \times 5.7$) per cent	Ash content* per cent	Moisture content per cent
Durum	12.0	0.53	14.0
Hard red spring mill blend	12.0	0.40	10.3
Hard red spring Vesta	13.3	0.40	10.2
Hard red spring Mida	14.0	0.38	10.7

* Expressed on 13.5 per cent moisture basis.

from a mill blend, while the other two were from two different wheat varieties chosen because of differences in their gluten protein characteristics, particularly their dough mixing properties (Harris and Sibbitt, 1945). The durum sample was included for the same reason, since differences exist in some physico-chemical properties between durum and hard red spring wheat gluten (Harris and Johnson, 1940). These flours were unbleached, sound, and had been stored for some months

before using at 5°C. Their moisture, protein, and ash contents are given in Table I. The K_2SO_4 used was of reagent grade. The papain was prepared by Merck and Company.

Methods

The glutens were separated from doughs mixed in a Hobart C-10 laboratory mixer from flour with a suitable quantity of 0.1 per cent $NaH_2PO_4 + Na_2HPO_4$ solution of approximately pH 6.8. After mixing the doughs were covered with tap water and kept at a temperature of 30°C. for one hour. The glutens were washed under a small stream of the phosphate solution until reasonably free from starch, and then immersed in this solution for 30 to 45 minutes at 30°C. The gluten was then removed from the liquid, pressed dry with the fingers and the quantity required divided into small (approximately 0.5 g.) pieces and placed in 0.1 *N* acetic acid.

Three methods of dispersing the washed, wet, crude gluten in 0.1 *N* acetic acid were studied in regard to their effects on the quantity of protein separated by different concentrations of K_2SO_4 at two temperatures. Method A consisted of dispersing the divided gluten in the Waring Blendor for five minutes at low speed. Several hours were allowed for the foam to disintegrate before heating the dispersion. In method B, 250 ml. Erlenmeyer flasks containing the gluten pieces in acetic acid were shaken by hand at approximately 10 minute intervals for six hours, or until the dispersion appeared to be complete. Care was taken not to cause foaming. In method C, the individual glutens in acetic acid in 250 ml. Erlenmeyer flasks were gently agitated in a rotary shaker at 14 r.p.m. (Harris and Johnson, 1940) until dispersion was judged to be complete. Some differences were noted in the visual appearance of the dispersions: those prepared on the rotary shaker were more flocculent in appearance and on centrifuging tended to separate in two layers. Because of this fact these dispersions were not centrifuged before fractionation as is the usual practice in this laboratory in gluten investigations. In subsequent experiments the dispersions were centrifuged for 20 minutes in an International type SB centrifuge and the material thrown down discarded.

The dispersions were next heated to 96°C. for 10 minutes to inactivate the adsorbed proteases, as described by Olcott, Sapirstein, and Blish (1943). These workers pointed out that changes in viscosity of gluten dispersions in acetic acid on standing are caused by adsorbed

flour proteinase rather than by denaturation of the protein by acetic acid. They also stated that little change was induced in the solubility characteristics of the gluten proteins by the heat treatment. After cooling to room temperature, the dispersions were diluted with an equal weight of 95.0 per cent alcohol, and the required amount of K_2SO_4 added with thorough agitation. The temperature of the dispersions was then lowered to the desired level. At first the salt was added in the dry form, but later a 10 per cent solution in distilled water was used, a suitable adjustment in the concentration being made to take care of the added volume of liquid. It was found that the use of the K_2SO_4 solution increased the speed of protein separation, and it appeared to be complete after eight hours in the cooler.

After separation of the protein had occurred the dispersions were placed in centrifuge bottles, centrifuged for 20 minutes, and the supernatant liquid decanted from the residual protein. The protein content of the decantate was determined, and this value subtracted from the original protein concentration yielded the quantity of protein removed. The other fractions were determined in a similar manner.

A suitable refrigerating unit was utilized for cooling the dispersions. This apparatus has a temperature range of $+20^\circ$ to $-20^\circ C.$ and controls the temperature of the liquids under investigation well within $\pm 1^\circ C.$, particularly in the lower temperature ranges. The capacity was sufficient to accommodate six 500 ml. flasks as well as a 250 ml. flask containing acetic acid-ethyl alcohol solution with a thermometer to indicate temperature levels in the dispersions.

RESULTS AND DISCUSSION

The data secured will be presented in the form of figures since these are easier to interpret than the numerical values themselves, and precise results cannot be reproduced in different laboratories.

Effect of Dispersion Method

Fig. I shows the results obtained from this preliminary investigation of the influence of dispersion method on protein fractionation. The "fractions" are arbitrarily defined as the quantities of protein removed from the dispersions at temperatures of 18° and $5^\circ C.$ and the residue remaining in colloidal solution after the separation of fraction 2, respectively. It is quite evident that the effect of method of dispersal is

insignificant and that any of the three methods could be used without changing the results. Method A, dispersal with the Waring Blender, was selected as the method employed in subsequent investigations as it fitted into the daily laboratory routine much better than the other

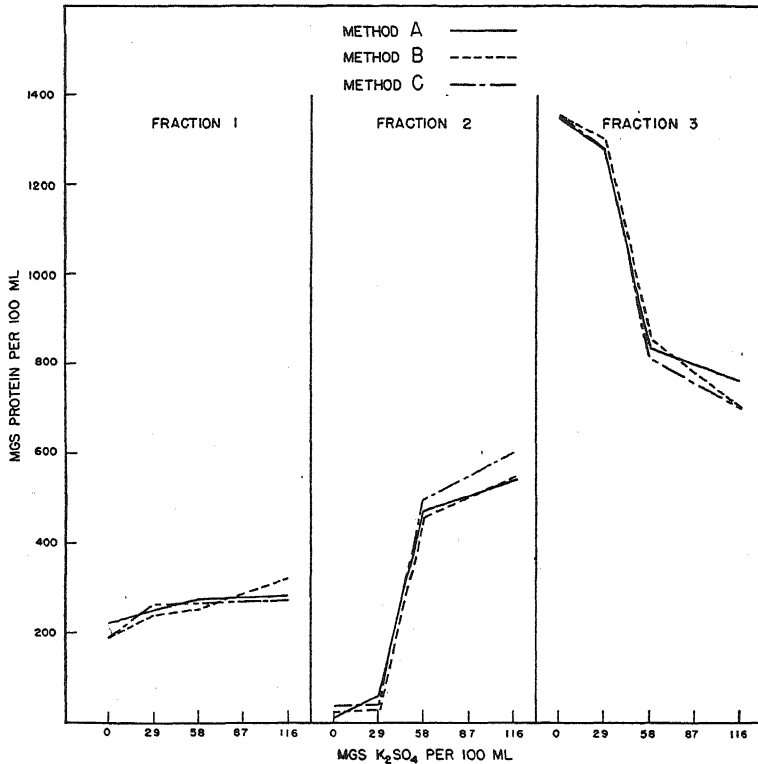


FIG. 1

Effects of Dispersion Method and K_2SO_4 Concentration on Thermal Protein Fractionation

two techniques. Apparently no significant amount of protein denaturation was caused by the foaming of the liquid during dispersion in the Blender. The influence of temperature is very marked on the quantity of protein removed as well as upon the physical appearance and characteristics of the fractions. Fraction 1 resembled the original gluten and corresponded to the classical "glutenin" component. Fraction 2

was less firm than fraction 1 and was more gelatinous in appearance. Fraction 3 was not separated but there is little doubt in view of the results from other studies that it would correspond with the description given by Harris and Bailey (1937).

Effect of K_2SO_4 Concentration

The effect of K_2SO_4 concentration is particularly interesting. Fraction 1 was little affected, but for fraction 2 after the lowest concentration employed, 29 mg. per 100 ml., there was a sharp rise in the quantity of protein removed. This increase tended to level off after 58 mg. of K_2SO_4 per 100 ml., but there was still a noticeable rise at the highest concentration used. Fraction 3, of course, varied inversely with the quantity of protein removed and decreased with increased K_2SO_4 treatment. These data emphasize the importance of K_2SO_4 concentration when employing thermal fractionation methods with acetic acid-ethyl alcohol dispersions of gluten. At low salt concentration, the greater proportion of the protein would be precipitated in fraction 3 and would not be removed at temperatures above 5°C. A relatively high K_2SO_4 concentration, on the other hand, throws out an increased quantity of fraction 2 and a reduced proportion of fraction 3 is then available. Little information regarding a gluten protein fraction is yielded by specifying fractionation temperature without also designating the K_2SO_4 concentration used. Some fractions may be shifted from one temperature zone to another by varying the salt concentration.

Effect of Wheat Variety

In the next experiment three wheat flours were used, two representing hard red spring wheat varieties while the third was milled from a durum wheat. Three concentrations of K_2SO_4 were again used, but the proportion of protein removed at temperatures of 18°, 14°, 10°, 6°, -5°, and -10°C. was determined. The total amount, as per cent of total dispersed protein, progressively removed with increased K_2SO_4 concentration is shown in Fig. 2. A marked difference in the results secured from the first experiment is evident in the quantity of protein removed at 18°C. This discrepancy was caused by the different treatments of the dispersions prior to fractionation. In the first experiment, the dispersions were not centrifuged before fractionating, and some of the protein removed before fractionation in the second experiment came

down at 18°C. when centrifugation was first employed. This variation in results emphasized the sensitivity of the method to changes in technique.

At the lowest concentration of K_2SO_4 , 30 mg. per 100 ml. of dispersion, no significant amount of protein was removed from any dispersion until the temperature had reached $-5^\circ C$. Amounts ranging from approximately 28 per cent to 16 per cent of the total were precipitated

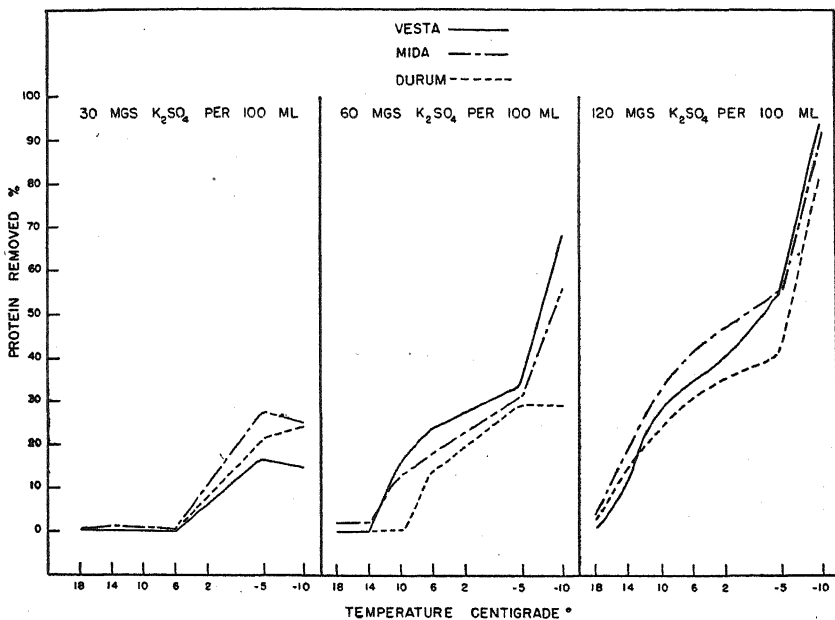


FIG. 2

Per Cent of Total Dispersed Protein Removed at Different Temperatures by Various Concentrations of K_2SO_4

at this temperature. Further lowering of the temperature had little effect in causing additional separation of protein. The dispersion with Mida wheat gluten gave the highest values and Vesta gave the lowest. At the next highest concentration, 60 mg. per 100 ml., more protein was removed, the precipitation commencing at 18°C. for Mida gluten and progressively continuing to reach a maximum of 68.5 per cent of the total dispersed protein at $-10^\circ C$. The durum protein, however,

was only 29 per cent accounted for at this temperature, and there is little doubt of the significance of this difference. This dispersion yielded low results throughout this K_2SO_4 concentration and these might indicate some difference between the wheats in the relative sizes of their protein particles in dispersion. It might be surmised that the durum wheat micelles were smallest in dispersion and Vesta micelles the largest with Mida having micelles intermediate in size. The dispersion possessing the largest particles would be expected to have its protein the most readily removed while the reverse would be true for the dispersion with the smallest micelles present. This hypothesis is in agreement with conclusions reached by Harris and Johnson (1940) when studying the viscosity of gluten dispersions in 10 per cent sodium salicylate.

When 120 mg. of K_2SO_4 were used there was almost a linear relationship between temperature and per cent of total protein removed. At $-10^\circ C$. 94 per cent of the Vesta protein had been removed, and 91 per cent of the Mida. The durum was again somewhat lower with 81.5 per cent precipitated. The authors are inclined to doubt if any significant differences are shown between the different flour gluten behaviors at this salt concentration, and the three curves can be considered as identical for all practical purposes. There is little indication of distinct protein "fractions" being removed at any temperature included in this experiment, except possibly at the lowest salt concentration at $-5^\circ C$. Rather there is a progressive increase in the proportion of total protein removed with a decrease in temperature, this increase being accelerated by increasing the amount of K_2SO_4 present. Apparently the dispersed gluten can be separated in the form of almost innumerable fractions corresponding to results secured by McCalla and Rose (1935) from sodium salicylate gluten dispersions.

Effect of Papain

Balls and Hale (1938) have pointed out the similarity between the flour proteases and papain. Accordingly, papain was used to obviate the tedious process of preparing flour protease. Harris and Johnson (1939) described the effect of addition of various enzymes to flour doughs on the relative quantities of protein removed from sodium salicylate dispersions of gluten by successive additions of $MgSO_4$. It was found that papain in particular markedly decreased the protein

removed as the initial fraction, and this was ascribed by the authors to a reduction in micellar size effected by the enzyme. The present experiment was undertaken to ascertain if a similar effect would be noticeable in a thermal fractionation of acetic acid-ethyl alcohol dispersions containing small amounts of K_2SO_4 .

The enzyme was incorporated in the dough in the form of a water suspension. When the highest concentration of enzyme was employed it was found necessary to use the method of Swanson and Andrews

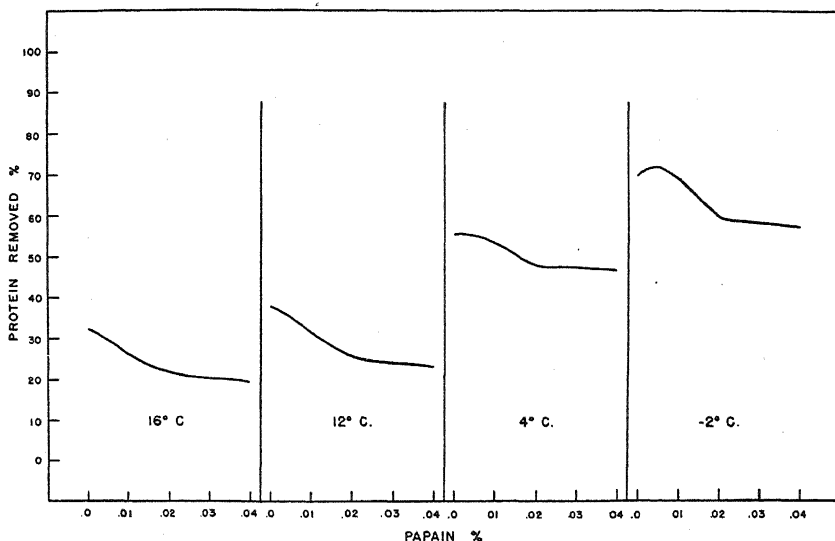


FIG. 3

Effect of Papain on the Per Cent of Total Dispersed Protein Removed at Different Temperatures

(1945) to separate the gluten from the dough. This technique entails the use of the Waring Blendor to disperse the dough in distilled water. The gluten is separated in the form of a foam phase and may be removed from the surface of the liquid. Upon centrifuging the gluten is obtained as a supernatant layer. A few minutes washing in the usual manner is all that is required to complete the separation of the gluten from the starch.

The data secured from the papain-treated doughs are shown in Fig. 3. Four protein fractions were determined for the four temperatures

shown. The effect of papain in decreasing the per cent of dispersed protein removed at the different temperatures is very evident. At the lowest temperature, $-2^{\circ}\text{C}.$, approximately 30 per cent of the protein is still dispersed, while at a papain concentration of 0.04 per cent, about 42.5 per cent of the protein is not accounted for. These results probably indicate reduction in protein micelle size with increasing severity of enzyme treatment as postulated by Harris and Johnson (1939).

SUMMARY

The method of gluten dispersion in 0.1 *N* acetic acid had no significant influence upon the proportions of gluten protein fractionated at 18° and $5^{\circ}\text{C}.$ from acetic acid-ethyl alcohol dispersions containing small quantities of K_2SO_4 . The salt concentration had little effect upon the quantity of protein removed at the higher temperature, but did markedly increase the protein precipitated at $5^{\circ}\text{C}.$ The quantity of protein remaining in solution was decreased by increasing K_2SO_4 concentration. "Fractions" may be shifted from one temperature level to another by altering the salt concentration.

No marked general differences in fractionation behavior were found among three wheat sorts, although the dispersion prepared from durum wheat gluten yielded lower proportions of precipitated protein at some of the temperatures employed. There was little evidence to support the contention that distinct protein "fractions" were removed at any of the six temperatures used in this section of the investigation, but instead a gradual increase in the proportion of total protein removed with decrease in fractionation temperature. Increased concentration of K_2SO_4 accelerated this precipitation.

Papain decreased the per cent of dispersed protein separated at the various temperatures. The results would seem to indicate reduction in protein micelle size with increasing severity of enzyme treatment.

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Mechanism of the Butyl Alcohol Fermentation with Heavy Carbon Acetic and Butyric Acids and Acetone *

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INTRODUCTION

Probably no bacterial fermentation has been studied more extensively with the object of determining the mechanism of its reaction than the butyl alcohol fermentation. Nevertheless, our understanding of this fermentation is very inadequate. Admittedly, a rather complete series of reactions may be written which are adaptable to the observed quantitative relationships of the end products, but on critical examination there is not adequate evidence for them.

The initial cleavage of glucose is suggested in most of the schemes to give rise to methyl glyoxal. Some of the schemes were written prior to knowledge of the role of phosphoglyceric acid as an intermediate in hexose metabolism of yeast and as a consequence this compound was not taken into consideration. Pett and Wynne (1) and Osburn, *et al.* (2) have isolated methyl glyoxal from the fermentation. However, this compound has been found to be very toxic to the butyl alcohol bacteria by Johnson, *et al.* (3) and its role as an intermediate is doubtful. It is noteworthy that Stone and Werkman (4) were unable to detect formation of phosphoglyceric acid with the butyl alcohol bacteria although they were successful with other bacteria. Until recently there has been no proof that phosphorylation plays a part in the fermentation. Koepsell and Johnson (5) have now demonstrated with a cell-free enzyme from *Clostridium butylicum* that phosphate is essential for at least one reaction, the conversion of pyruvate to H_2 , CO_2 , and acetic acid.

In most bacterial fermentations pyruvate is generally accepted as a key compound. However, in the butyl alcohol fermentation there is disagreement regarding the role of pyruvate. Primarily, the view that pyruvate is not an intermediate is maintained by Kluyver (6) and Simon (7) because neither butyl alcohol nor butyric acid is formed

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in considerable quantity from pyruvate by *Cl. acetobutylicum*. However, it has been pointed out by Johnson, *et al.* (3) that because of the degree of oxidation of pyruvate a substantial formation of the 4-carbon compounds is not to be expected. In the closely related fermentation by *Cl. butylicum*, pyruvate is converted to butyric acid, and evidence has been presented that pyruvate is an intermediate in glucose dissimilation (Osburn, *et al.*, 2, 8). It seems very improbable that these two fermentations possess essentially different mechanisms, since they are practically identical except that acetone is formed in one and isopropyl alcohol in the other.

The mechanism of formation of butyric acid and butyl alcohol is uncertain. Since its early introduction by Fitz in 1876 (12), the concept of the condensation of acetaldehyde to acetaldol and its subsequent conversion to butyric acid and butyl alcohol has found many advocates (9, 10, 6, 11), despite the fact that in repeated attempts acetaldehyde has never been detected in the butyl alcohol fermentation (13, 14, 2). The results of Neuberg and Arinstein (15) constitute a possible exception, but the purity of their culture is questioned (14). Acetaldol is very toxic to the fermentation and when added in dilute solution remains unchanged (3, 11).

Other investigators have favored a scheme involving pyruvic aldol (15, 14). Peldan (14) has observed that the calcium ion stimulated butyrate formation from pyruvate. He believes that calcium facilitates formation of the pyruvic aldol.

There is general agreement concerning the mechanism of acetone formation. Acetic acid when added to fermentations produces a large increase in acetone. It is believed that the acid condenses to acetoacetic acid and that this compound decarboxylates to acetone (16, 17, 8). Johnson, *et al.* (3) and Davies (18, 19) have studied the enzyme that decarboxylates acetoacetic acid.

Ethyl alcohol is almost uniformly proposed to arise by reduction of acetaldehyde and butyl alcohol by reduction of butyric acid. In the normal fermentation there is a simultaneous disappearance of butyrate and increase in butyl alcohol at the time that solvent formation occurs (13, 20).

It is apparent that most of the information concerning the butyl alcohol fermentation has been gained by adding a proposed intermediate compound to the fermentation and judging the pathway of the added components by changes in the end products. The method is of course not strictly valid; a simple addition of a hydrogen acceptor such as methylene blue will change the relative proportion of end products. The advent of C^{13} as a tracer element presented a technique by which these assumptions could be largely eliminated. Experiments have therefore been conducted to determine the fate of labeled acetate, butyrate, and acetone in corn mash fermentations by *Cl. acetobutylicum* and *Cl. butylicum*.

METHODS

The fermentations were conducted as described by Brown, *et al.* (22), except for the medium which is given in Tables I, II, and III. The phosphate and acid mixture

TABLE I

Distribution of C¹³ of CH₃·C¹³OOH among the Products of Fermentation by the Butyl Alcohol Bacteria

Medium: 100 ml., 3.75 g. corn meal, 5.77 mM K₂HPO₄, 6.15 mM CH₃·C¹³OOH; in addition 0.1 g. of Bacto yeast extract in case of *Cl. butylicum*.

	<i>Cl. acetobutylicum</i>				<i>Cl. butylicum</i>			
		C ¹³ in excess of normal	C ¹³ in excess of normal	Recovery of C ¹³ of added acetic acid		C ¹³ in excess of normal	C ¹³ in excess of normal	Recovery of C ¹³ of added acetic acid
	mM	per cent	mM	per cent	mM	per cent	mM	per cent
Acetone	5.04	0.72	0.109	15				
Isopropyl alcohol					5.38	0.84	0.136	19
Butyl alcohol	8.19	1.25	0.410	55	6.37	1.21	0.308	43
Ethyl alcohol	1.89	0.54	0.020	3	0.23	0.70	0.003	0.4
Acetic acid	4.40	0.43	0.038	5	1.06	0.24	0.005	1
Butyric acid	0.75	0.70	0.021	3	3.36	0.17	0.023	3
Formic acid	0.36	0.14	0.002	0.3	0.10	0.10	0.000	0.0
CO ₂	35.40	0.38	0.135	18	35.00	0.53	0.186	26
Filtrate	15.60*	0.08	0.013	2	34.40*	0.05	0.017	2
Solids	15.10*	0.05	0.008	1				
Totals			0.756	101	Totals		0.678	94
Acetic acid added	6.15	6.09	0.749		6.15	5.87	0.722	

* mM of carbon in fraction obtained from fermented medium following removal of volatile compounds.

was added at the time of inoculation of the corn mash, which had been cooked in the autoclave for three hours. The adjustment of the pH with phosphate was according to the procedure of Osburn, *et al.* (8) so that free acid was available for reduction in the fermentation.

The general procedure of fractionation of the products was the same as previously described (21, 22). Modification of the procedure was required in the fermentation by *Cl. butylicum* to which CH₃·C¹³O·CH₃ was added so that the acetone could be separated from the isopropyl alcohol. The acetone and alcohols in the alkaline distillate were subjected to the iodoform reaction (23). Acetone was thus converted to iodoform and acetic acid, while the alcohols were unchanged. The iodoform was filtered off after titration of the excess iodine with thiosulfate, and the alcohols were separated from the acetic acid by one half volume distillation of the neutralized filtrate. The acetic acid in the residue was then obtained by acid steam distillation.

TABLE II

Distribution of C^{13} of $CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot C^{13}OOH$ among the Products of Fermentation by the Butyl Alcohol Bacteria

Medium: 45 ml., 1.70 g. corn meal, 2.15 mM KH_2PO_4 , 2.43 mM $CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot C^{13}OONa$, in addition 0.05 g. of Bacto yeast extract in case of *Cl. butylicum*.

	<i>Cl. acetobutylicum</i>				<i>Cl. butylicum</i>			
		C^{13} in excess of normal	C^{13} in excess of normal	Recovery of C^{13} of added butyric acid		C^{13} in excess of normal	C^{13} in excess of normal	Recovery of C^{13} of added butyric acid
	mM	per cent	mM	per cent	mM	per cent	mM	per cent
Acetone	1.47	0.04	0.002	2				
Isopropyl alcohol					1.71	0.08	0.004	3
Butyl alcohol	4.98	0.51	0.102	84	4.70	0.53	0.100	83
Ethyl alcohol	0.68	0.04	0.001	1	0.19	—	—	—
Acetic acid	2.65	0.02	—	—	1.29	0.06	0.002	2
Butyric acid	1.03	0.38	0.016	13	1.47	0.07	0.004	3
CO ₂	16.50	0.01	—	—	14.90	0.01	—	—
Filtrate	17.20*	0.02	—	—	19.90*	-0.01	—	—
Solids	17.00*	0.00	—	—	22.00*	-0.01	—	—
Totals			0.121	100	Totals		0.110	91
Butyric acid added	2.43	1.24	0.121		2.43	1.24	0.121	

* mM of carbon in fraction obtained from fermented medium following removal of volatile compounds.

Combined carbon of the iodoform and acetic acid represents that of the acetone. The acetone-free alcohols were then oxidized with dichromate and the resulting acetone and acids were separated as previously described (22).

Additional fractionation was necessary in those fermentations in which the C^{13} in the butyl and ethyl alcohols and in the acetic and butyric acids was to be determined individually. The procedure was based on separation of the acids by azeotropic distillation with benzene and toluene as described by Schickltanz, *et al.* (24). Since the alcohols were oxidized to acids, the same procedure could be applied in their separation. In practice, the rate of distillation was followed by titration of the acid in the benzene, and when the rate reached a low value the toluene distillation was started. After tritration, the water solution of the salts was separated from the benzene or toluene, and the acids were recovered by steam distillation and determined by partitioning (25).

In our hands the separation of acetic and butyric acids by the azeotropic distillation was not quantitative. Usually from the fermentation acids, the acid obtained in the

TABLE III

Distribution of C^{13} of $CH_3 \cdot C^{13}O \cdot CH_3$ among the Products of Fermentation by the Butyl Alcohol Bacteria

Medium: 60 ml., 2.25 g. corn meal, 1.69 mM of $CH_3 \cdot C^{13}O \cdot CH_3$; in addition 0.07 g. of Bacto yeast extract in case of *Cl. butylicum*.

	<i>Cl. acetobutylicum</i>				<i>Cl. butylicum</i>			
		C^{13} in excess of normal	C^{13} in excess of normal	Recovery of C^{13} of added acetone		C^{13} in excess of normal	C^{13} in excess of normal	Recovery of C^{13} of added acetone
	mM	per cent	mM	per cent	mM	per cent	mM	per cent
Acetone	3.72	0.26	0.029	81	0.31	0.23	0.002	5
Isopropyl alcohol					3.85	0.28	0.032	89
Butyl alcohol	4.48	0.01	—	—	5.21	0.01	—	—
Ethyl alcohol	1.05				0.11			
Acetic acid	1.49	0.01	—	—	0.40	0.02	—	—
Butyric acid	0.26				0.18			
CO ₂	21.20	0.02	—	—	18.20	0.00	—	—
Filtrate	27.7*	0.00	—	—	30.3*	-0.01	—	—
Solids	30.6*	0.00	—	—	20.7*	-0.01	—	—
Totals			0.029	81	Totals		0.034	94
Acetone added	1.69	0.72	0.036		1.69	0.72	0.036	

* mM of carbon in fraction obtained from fermented medium after removal of volatile compounds.

benzene fraction was almost pure acetic acid, frequently being 99 or 98 per cent, but sometimes as low as 90 per cent; the remainder was butyric acid. The acid in the toluene fraction was never pure butyric, usually being about 90 per cent butyric and 10 per cent acetic acids. When the first part of the toluene distillation was excluded the purity of the butyric acid was improved. This procedure was followed when the acid was to be used in subsequent fermentations as a source of C^{13} butyric acid.

It was not necessary to have the pure acids in order to determine their individual C^{13} content. From the C^{13} content of the mixtures of acids obtained in the benzene and toluene fraction, the C^{13} was calculated by simultaneous equations, cf. Addendum.

In addition to determining the C^{13} content of the whole molecule, three of the compounds, acetone, isopropyl alcohol, and butyl alcohol, were degraded to determine the location of the labeled carbon within the carbon chain. The procedure and typical examples of results from the degradation of butyl alcohol (as butyric acid) has been presented elsewhere, Wood *et al.* (21). The degradation of acetone and isopropyl alcohol, after oxidation to acetone, was done by the iodoform reaction (23, 22). The

products of this reaction, CHI_3 and acetic acid, were separated and their C^{13} content determined. The CHI_3 represents the methyl group, the acetic acid both the methyl and carbonyl groups of the acetone.

The purity of the respective fractions which were separated from the fermentation was checked by determining the agreement between the total carbon and the carbon calculated from the products which were determined to be present in the fraction. The agreement was usually good. All C^{13} values reported in this paper are expressed in per cent in excess of the normal complement of C^{13} (1.09 per cent). The mM of C^{13} in excess of normal was calculated from the total carbon in the product or fraction and the per cent C^{13} .

The heavy carbon butyric acid and acetone, which were added to the butyl fermentations were products isolated from fermentations to which $\text{CH}_3\cdot\text{C}^{13}\text{OOH}$ had been added. The $\text{CH}_3\cdot\text{C}^{13}\text{OOH}$ was synthesized from methyl magnesium bromide and C^{13}O_2 by the Grignard reaction.

RESULTS AND DISCUSSION

The most informative data from these experiments are the per cent excess C^{13} and per cent recovery of the added C^{13} in the respective products. Both data must be considered in conjunction with the other in order to give the most complete information. A product could be formed exclusively from the added heavy compound, and thus have a high per cent excess C^{13} , yet, if the yield were low, the proportion of the total added C^{13} recovered in this product would be low. Thus, the amount of C^{13} recovered shows the proportion of the added C^{13} compound which was shunted into the product, whereas the per cent C^{13} is an indication of the fraction of its total carbon coming from the added C^{13} compound on the one hand, and from the corn starch on the other.

From the standpoint of interpreting mechanism it is important to know that the C^{13} compound is used without previous change to some completely unrelated compound. Conceivably, the added compound could first be oxidized to CO_2 and then the CO_2 be fixed in the product. This possibility has been studied by the addition of C^{13}O_2 to the butyl fermentation [Slade, *et al.* (26) and Brown, *et al.* (22)], and it has been found that C^{13}O_2 is fixed only in formic, lactic, and pyruvic acids. The possibility has frequently been suggested that added compounds are first converted to a C_6 compound, such as glucose and then in turn to the products of fermentation. If this occurred with $\text{CH}_3\cdot\text{C}^{13}\text{OOH}$ and by a simple union, it would result in four types of C_6 chains: $\text{C}\cdot\text{C}^{13}\text{C}\cdot\cdot\text{C}^{13}\cdot\text{C}\cdot\text{C}^{13}$ or $\text{C}\cdot\text{C}^{13}\cdot\text{C}^{13}\cdot\text{C}\cdot\text{C}\cdot\text{C}^{13}$ or $\text{C}^{13}\cdot\text{C}\cdot\text{C}\cdot\text{C}^{13}\cdot\text{C}\cdot\text{C}^{13}$ or $\text{C}\cdot\text{C}^{13}\cdot\text{C}\cdot\text{C}^{13}\cdot\text{C}^{13}\cdot\text{C}$. If these chains were fermented according to the classical

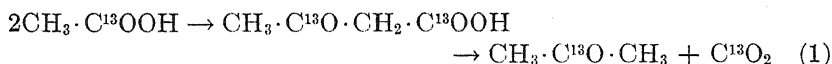
schemes, $\text{CH}_3 \cdot \text{C}^{13}\text{O} \cdot \text{COOH}$ and $\text{C}^{13}\text{H}_3 \cdot \text{CO} \cdot \text{C}^{13}\text{OOH}$, or $\text{CH}_3 \cdot \text{C}^{13}\text{O} \cdot \text{C}^{13}\text{OOH}$ and $\text{C}^{13}\text{H}_3 \cdot \text{CO} \cdot \text{COOH}$, or $\text{C}^{13}\text{H}_3 \cdot \text{CO} \cdot \text{COOH}$ and $\text{C}^{13}\text{H}_3 \cdot \text{CO} \cdot \text{C}^{13}\text{OOH}$, or $\text{CH}_3 \cdot \text{C}^{13}\text{O} \cdot \text{COOH}$ and $\text{CH}_3 \cdot \text{C}^{13}\text{O} \cdot \text{C}^{13}\text{OOH}$ would be formed. In only the last case would the conversion products be expected to have the same relationship of C^{13} in the products as in a more direct conversion of $\text{CH}_3 \cdot \text{C}^{13}\text{OOH}$.

Experiments with $\text{CH}_3 \cdot \text{C}^{13}\text{OOH}$

The distribution of the C^{13} of $\text{CH}_3 \cdot \text{C}^{13}\text{OOH}$ among the products of fermentation is shown in Table I.

The most striking point revealed by the data is that in both, the fermentation by *Cl. acetobutylicum* and *Cl. butylicum*, the major portion of the added C^{13} acetate was converted into butyl alcohol, 55 per cent by *Cl. acetobutylicum* and 43 per cent by *Cl. butylicum*. This observation is the more surprising because it has been repeatedly observed that there is an increase in acetone or isopropyl alcohol which is equivalent to the added acetic acid (16, 17, 8). The conclusion had therefore been made that the added acetate is almost quantitatively converted to acetone or isopropyl alcohol. It is now clear that this conclusion is incorrect; a large part of the acetate is converted to butyl alcohol.

A substantial part of the acetic acid is converted to acetone and isopropyl alcohol, 15 per cent of the added C^{13} of acetate being recovered in acetone and 19 per cent in the isopropyl alcohol. According to the theory that the acetic acid condenses to acetoacetic acid and then is converted to acetone and CO_2 , the recovery of C^{13} in the CO_2 should be at least equivalent to that of the acetone.



In both fermentations the recovery of C^{13} in the CO_2 was somewhat higher, 18 and 26 per cent, than from the acetone and isopropyl alcohol. It therefore appears that there may be a small additional formation of CO_2 from acetic acid other than by decarboxylation of acetoacetic acid (Eq. 1). No mechanism for such a conversion has been suggested or is at present obvious.

It is interesting that only 5 per cent of the C^{13} of the acetate was recovered in the residual acetate in one fermentation, and 1 per cent in the other, whereas the residual acetate amounted to 4.40 mM (71 per cent) and 1.06 mM (17 per cent) of the 6.15 mM of the added C^{13}

acetate. The residual acetic acid contained only 0.43 and 0.24 per cent C^{13} . A plausible explanation for these observed changes is that the acetate used at first would contain approximately 6 per cent C^{13} , and as the sugar was fermented the C^{13} acetate would be diluted by C^{12} acetate produced from the corn starch. Since the concentrated C^{13} would be used at first and that which was left would be constantly diluted, it is obvious that the residual acetate would be low in C^{13} .

Since butyric acid is generally believed to be a precursor of butyl alcohol, it might, on first thought, be expected that these two compounds would have a very similar C^{13} content. Actually there is a wide divergence, butyric acid being 0.7 and 0.17 and butyl alcohol 1.25 and 1.21 per cent C^{13} . Since the acetic acid is continuously being diluted, if butyric acid is formed first from the acetic acid and is then converted to butyl alcohol, it is obvious that the butyric acid which replaces the acid converted to butyl alcohol must have less C^{13} than the butyl alcohol.

The C^{13} in the ethyl alcohol, 0.54 and 0.70 per cent, is less than in the butyl alcohol. This may indicate that the ethyl alcohol is not formed from acetate to the same extent as butyl alcohol or, on the other hand, that it is formed at a different phase or time in the fermentation. The evidence is at least clear that acetic acid is converted to ethyl alcohol.

There was some C^{13} in the formic acid which was probably formed by reduction of the CO_2 .

Experiments with $CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot C^{13}OOH$

There were two primary purposes in the experiments with C^{13} -*n*-butyric acid. One was to confirm the conversion of butyric acid to butyl alcohol. The second was to determine the reversibility of the reactions leading to formation of butyric acid. It was to be expected that the C^{13} of the butyric acid would be found in other products of the fermentation if the reactions were sufficiently reversible to reach a common precursor.

The results, Table II, show that the major portion of the butyric acid was converted to butyl alcohol: 84 per cent of the C^{13} was in the butyl alcohol with *Cl. acetobutylicum* and 83 per cent with *Cl. butylicum*.

Apparently the butyric acid was not converted solely to butyl alcohol. Although the results for ethyl alcohol are not considered very significant because of the indirect method used in the determination

of its C^{13} content, the values for the acetone from *Cl. acetobutylicum* and for the isopropyl alcohol and acetic acid from *Cl. butylicum* are well beyond the limits of error of the mass spectrometer. The only possible sources of error are that the fractions were contaminated with an unknown C^{13} compound (none was detected by total carbon determination) or the original butyric acid added to the fermentation was not pure. By the partition method the original butyric acid was determined as 98 per cent *n*-butyric acid and 2 per cent propionic acid, and the total carbon checked with these determinations. Accordingly, the propionic acid which was added as a contaminant of the butyric acid was 0.05 *mM*. This contamination has been ignored in our calculations. If propionic acid was present, the majority of it would very likely be reduced to propyl alcohol, and even if all was converted to one compound it could account for only 0.001 *mM* of excess C^{13} . The results seem, therefore, to be conclusive that there was a small conversion of butyrate to compounds other than butyl alcohol. Whether this conversion is the result of reversal of the reactions which lead to butyrate synthesis or the result of other reactions is uncertain. If the conversion is due to reversibility, the equilibrium must be well toward the butyric acid since only a small per cent of the C^{13} butyric acid was found in the acetone and acetic acid.

Experiments with $CH_3 \cdot C^{13}O \cdot CH_3$

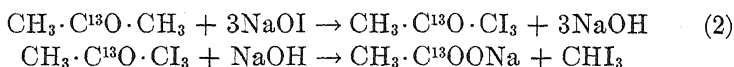
These experiments (Table III) are of principal interest with respect to the fermentation by *Cl. butylicum*. The purpose was to show, first that the added acetone is converted to isopropyl alcohol, and second to obtain information as to whether or not the acetone is an intermediate in isopropyl alcohol formation. It is probable that if acetone is formed as an intermediate there would be mixing of this acetone (which contains no excess C^{13}) with the C^{13} acetone. Because of this interchange, the acetone remaining at the end of the fermentation should have a C^{13} content lower than that of the added acetone. The results are in agreement with this expectation. The original acetone contained 0.72 per cent C^{13} , whereas the acetone isolated from the fermentation contained 0.23 per cent. The per cent C^{13} in the isopropyl alcohol was 0.28, thus quite close to that of the acetone. This observation suggests that the acetone and isopropyl alcohol are in reversible equilibrium. Langlykke, *et al.* (27) have detected acetone in fermenta-

tions with *Cl. butylicum*. The evidence therefore seems fairly complete that acetone may be intermediate in the formation of isopropyl alcohol.

The experiments (Table III) are not satisfactory for demonstrating a reversible reaction, *e.g.*, between acetone and acetic acid, because the C^{13} content of the original acetone was not sufficient to permit the detection of small conversions. Most of the products appear to have contained a slight but not significant excess of C^{13} . Since the recovery of the C^{13} of the acetone was somewhat low, a secondary conversion is indicated by this result as well.

Degradation of C^{13} Compounds Isolated from the Butyl Fermentations

Acetone and isopropyl alcohol were routinely degraded in the analysis of each fermentation by the iodoform reaction:



This procedure was followed because it offered the best assurance that the isolated carbon was exclusively from acetone or isopropyl alcohol which had been converted to acetone. The combination of properties: volatility from alkaline solution, stability to dichromate oxidation and formation of only iodoform and acetic acid by the iodoform reaction, are practically limited to acetone. The values given in the tables are for the combined C^{13} of iodoform and acetic acid obtained from the acetone. The results of these degradations are not given in table form because they were uniformly the same. There was no excess C^{13} in the iodoform, and all of the excess C^{13} was exclusively in the acetic acid. These results prove that when $\text{CH}_3 \cdot C^{13}\text{OOH}$, $\text{CH}_3 \cdot C^{13}\text{H}_2 \cdot \text{CH}_2 \cdot C^{13}\text{OOH}$, or $\text{CH}_3 \cdot C^{13}\text{O} \cdot \text{CH}_3$ was added to fermentations by *Cl. acetobutylicum* or *Cl. butylicum* the acetone or isopropyl alcohol formed in these fermentations contains C^{13} exclusively in the carbonyl group of the acetone and the carbinol group of the isopropyl alcohol (Eq. 2).

The degradation of butyl alcohol (after conversion to butyric acid) has been described in detail and the results presented (21). The degradation given in Experiment 3 of that report was on the butyl alcohol from the fermentation by *Cl. butylicum* (Table I). The degradation from experiment 4 was on butyl alcohol from a fermentation by *Cl. acetobutylicum* similar to that described in Table I, except that the added $\text{CH}_3 \cdot C^{13}\text{OOH}$ contained only 2.56 per cent excess C^{13} . The es-

sential result of these degradations in so far as the present paper is concerned is to show that the C^{13} is exclusively in the carbinol and in the β carbons of the butyl alcohol. The concentration of C^{13} found in the carbinol and β carbons was 2.64 and 2.20 per cent respectively in Experiment 3, and 1.04 and 1.06 per cent in Experiment 4. It is, therefore, apparent that the C^{13} is approximately equally distributed in two positions of the 4 carbon chains. The difference found in Experiment 3 may have resulted from experimental error and no definite conclusion could be drawn concerning it. Degradations were also carried out on the butyl alcohol of the experiments described in Table II. Owing to an experimental difficulty, the results were not completely accurate, but the indications were that there was a similar distribution of C^{13} in these alcohols.

It is evident that the determination of the location of the C^{13} provides information of considerable significance for interpretation of the mechanism of the butyl alcohol fermentation. The proof that $CH_3 \cdot C^{13}O \cdot CH_3$ is formed in fermentations by *Cl. acetobutylicum* to which $CH_3 \cdot C^{13}OOH$ is added, and that $CH_3 \cdot C^{13}H(OH) \cdot CH_3$ is formed in similar fermentations by *Cl. butylicum* gives weight to the proposal that the acetone is formed by condensation of acetic acid to acetoacetic acid which is decarboxylated to acetone (Eq. 1). It is recognized, of course, that the transformation of the carboxyl of acetic acid into the the carbonyl of acetone, is not sufficient to prove that acetoacetic acid is the intermediate.

There are a number of observations which indicate that acetone and butyric acid may have a common precursor: (1) $CH_3 \cdot C^{13}OOH$ is converted in substantial yield to both acetone and butyl alcohol. (2) The position and concentration of the C^{13} in the molecules is such as to suggest a common origin of the compounds. (3) The addition of $CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot C^{13}OOH$ to the fermentations leads to formation of $CH_3 \cdot C^{13}O \cdot CH_3$. Observation (1) shows that there can be a common origin when acetic acid is added. It is uncertain whether this conversion occurs in a normal fermentation, and whether there is a common precursor subsequent to acetic acid such as acetoacetic acid. With regard to the second point the alternate positions of the labeled carbon in both the acetone and the butyl alcohol is suggestive of a common precursor as is the similarity of the concentration of C^{13} in the labeled carbon (2.14 and 2.52 for acetone, and 2.50 and 2.42 for butyl alcohol in the fermentations of Table I). The calculations of C^{13} concentrations

have been made on the assumption of an equal distribution of C^{13} in the two labeled carbons of the butyl alcohol. With regard to the third observation, while there is no information as to the mechanism by which the butyl bacteria convert butyric acid to acetone; in animal tissue the conversion is believed to occur through acetoacetic acid (28). It is conceivable that the same reaction occurs with the bacteria. In this connection it should be noted that Davies (18) could find no evidence that acetoacetic acid is reduced by cell suspensions of *Cl. acetobutylicum* which are fermenting glucose. Therefore this compound was considered an improbable precursor of butyrate.

Davies (18) also showed with cell suspensions of *Cl. acetobutylicum* that no acetone is formed when acetate is the only substrate. In combination with pyruvate or glucose, a rather high concentration of acetate was required to obtain maximum formation of acetone. Davies has suggested that acetoacetate formation involves both pyruvate and acetate. Acetopyruvic acid was considered an unlikely intermediate because it was toxic to the fermentation and was not attacked.

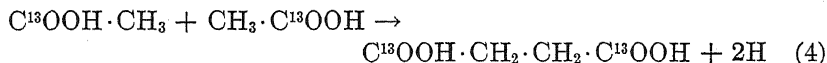
Results from the butyl alcohol degradation are of particular interest in this connection, especially if butyl alcohol and acetone are considered to have a common precursor. The implication is inherent in Davies' results that the acetic acid may react with pyruvate. If this combination of pyruvate and acetate was then converted through a common precursor to acetone and butyl alcohol, only half of the butyl alcohol would be of acetate origin. With acetate labeled in the carboxyl group the resulting butyl alcohol would contain C^{13} in only one position. The butyl alcohol degradation shows decisively that this is not the case. Furthermore, since the C^{13} is very nearly of the same concentration in the two positions, there apparently was no preferential formation of one part of the butyl alcohol molecule from starch carbon as opposed to acetate carbon. A possible error in this reasoning is that at some intermediate stage a symmetrical molecule was formed so that the labeled carbon became randomized in both halves of the molecule. The possibility of there being a symmetrical intermediate to a molecule which contains a methyl group on one end and a carbinol on the other is remote. The results indicate that the entire butyl alcohol molecule may be formed from acetate or a derivative of acetate. The same may be true for acetone. It is probable that the function of the glucose or pyruvate, as observed by Davies, is to supply the energy for the condensation of acetate.

There is one possible conversion of acetate which, if shown to occur with the butyl alcohol bacteria, would necessitate a modification of views. When the term acetate or a derivative of acetate is used, the implication is that reference is made to a C_2 -compound, such as acetaldehyde or acetyl phosphate. However, it is conceivable that the acetate is converted to pyruvate by the addition of formate as demonstrated with *Escherichia coli* by Utter, *et al.* (29):

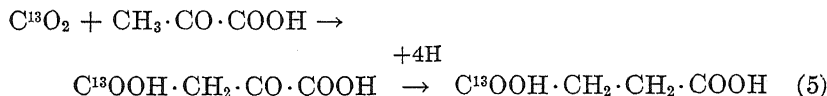


Or, the reaction might be the reverse of the conversion studied by Koepsell, *et al.* (5) with *Cl. butylicum* in which pyruvic acid was converted to acetic acid, CO_2 , and H_2 , and formic was not an intermediate. In any event, acetate would be converted to pyruvate, and the more direct utilization of acetate by C_2 conversion would not be realized. The conversion of acetate to pyruvate followed by pyruvate conversion to butyl alcohol likewise would account for the equal distribution of C^{13} in the carbinol and β carbons of the butyl alcohol.

The general significance of reaction (3) in biochemistry is not established at present. Conceivably, it could be the first step in acetate oxidation, the acetate being converted to pyruvate and then oxidized by a cycle such as that of Krebs. The only evidence that is available at present indicates the reaction did not occur significantly. Slade and Werkman (30) added $CH_3 \cdot C^{13}OOH$ to a fermentation of glucose by *Aerobacter*. They found the succinic acid to contain C^{13} in only the carboxyl groups and concluded that the conversion was, as follows:

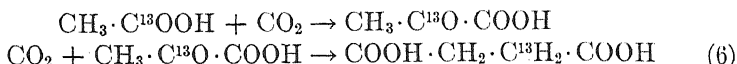


CO_2 is fixed in the carboxyl groups of succinic acid by *Aerobacter* presumably according to the reaction:



Accordingly, when $CH_3 \cdot C^{13}OOH$ was added to the fermentation, if pyruvate with C^{13} in the carbonyl group was formed from the acetate,

succinate should have resulted by the fixation reaction with C^{13} in the methylene group.



There was no indication of such a reaction.

SUMMARY

The mechanism of the butyl alcohol fermentation by *Cl. acetobutylicum* and *Cl. butylicum* has been investigated with the following labeled compounds: $CH_3 \cdot C^{13}OOH$, $CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot C^{13}OOH$, and $CH_3 \cdot C^{13}O \cdot CH_3$. The labeled compounds were added to corn mash fermentations; the products of fermentation were isolated and their C^{13} content was determined. The position of the labeled C^{13} within the carbon chain was determined in the butyl alcohol, acetone, and isopropyl alcohol.

Results with $CH_3 \cdot C^{13}OOH$

1. The C^{13} was recovered in butyl alcohol, acetone, isopropyl alcohol, butyric acid, acetic acid, ethyl alcohol, and CO_2 .
2. The butyl alcohol contained approximately 50 per cent of the added C^{13} . It is therefore obvious that added acetic acid is not converted quantitatively to acetone or isopropyl alcohol, as has been previously reported.
3. The labeled carbon in the butyl alcohol is in the carbinol and β -positions, and the concentration of C^{13} is approximately the same in these two positions. It is suggested that the molecule is synthesized from acetic acid or a derivative. There was no evidence of the preferential union of acetate with an intermediate compound from the starch.
4. The acetone or isopropyl alcohol accounted for 15 to 19 per cent of the added C^{13} . The labeled carbon was exclusively in the carbonyl and carbinol groups respectively.
5. The C^{13} recovery in the CO_2 was approximately the same in the acetone and isopropyl alcohol. This fact, together with established position of the C^{13} in the acetone and isopropyl alcohol, is in agreement with the suggestion that acetone is formed by decarboxylation of acetoacetic acid which is formed from acetate.

6. It is suggested that the butyric acid, butyl alcohol, and acetone may have a common precursor which is formed from acetate or a derivative of acetate.

Possible sources of error in the above suggestions are discussed.

Results with $\text{CH}_3 \cdot \text{C}^{13}\text{H}_2 \cdot \text{CH}_2 \cdot \text{C}^{13}\text{OOH}$

1. The C^{13} was recovered in butyl, ethyl and isopropyl alcohols, and acetic and butyric acids.

2. Approximately 85 per cent of the added C^{13} was present in the butyl alcohol. The labeled carbon was in the carbinol and β -positions. It is suggested that butyric acid is a precursor of butyl alcohol.

3. The conversion of butyric acid to acetic acid, acetone and isopropyl alcohol may be a reversible series of reactions through acetoacetic acid. The location of the labeled carbon in the acetone and isopropyl alcohol is in agreement with this proposal.

Results with $\text{CH}_3 \cdot \text{C}^{13}\text{O} \cdot \text{CH}_3$

1. The C^{13} was recovered in isopropyl alcohol. The presence of labeled carbon in other products could not be reliably detected.

2. Evidence is presented which indicates that acetone is an intermediate in the formation of isopropyl alcohol.

ADDENDUM

Example of calculations:

In the separation of the volatile acids the benzene fraction contained 95.3 per cent acetic acid and 4.7 per cent butyric acid, and the excess C^{13} of the carbon of the mixture was 0.05 per cent. The toluene fraction contained 12.2 per cent acetic acid and 87.8 per cent butyric acid, and the C^{13} was 0.36 per cent. The acid percentages are given as molar per cent, *i.e.*, *mM* per 100 *mM* of total acid. If X = the per cent excess C^{13} in the acetic acid and Y = the per cent excess C^{13} in the butyric acid, the following equations may be set up:

$$\begin{aligned}(95.3 \times 2)X + (4.7 \times 4)Y &= (95.3 \times 2 + 4.7 \times 4)0.05; \\ (12.2 \times 2)X + (87.8 \times 4)Y &= (12.2 \times 2 + 87.8 \times 4)0.36; \\ X &= 0.02 \text{ per cent excess } \text{C}^{13} \text{ in the acetic acid;} \\ Y &= 0.38 \text{ per cent excess } \text{C}^{13} \text{ in the butyric acid.}\end{aligned}$$

The procedure for determination of the C^{13} in the ethyl alcohol and butyl alcohol is more complicated. Although the ethyl alcohol on oxidation with dichromate yields acetic acid quantitatively, the butyl alcohol yields 0.033 moles of acetic, 0.120 moles of propionic and 0.752 moles of butyric per mole of butyl alcohol. Adjustment must therefore be made to the C^{13} of the acetic acid before the true C^{13} value of the acid of

ethyl alcohol origin is obtained. Furthermore, in the azeotropic distillation the propionic acid is distributed in both the benzene and toluene fractions. Correction must therefore be made for its presence in the two fractions before the true C^{13} value for acetic acid and butyric acid may be calculated.

These calculations have been made on the assumption that all the acetic and none of the butyric acid is in the benzene fraction. This assumption has been checked on a few fractions in which sufficient material was available for determination of two partition constants and found to be true. Furthermore, it has been assumed that the propionic acid comes from the α , β , and γ carbons of the butyl alcohol; the acetic from the β and γ carbons. Since the C^{13} in the butyl alcohol has been shown to be exclusively in the carbinol and β carbons and approximately in equal concentration (Wood, *et al.*, 21), the acetic acid of butyl alcohol origin would have the same per cent C^{13} as the whole butyl alcohol molecule, the propionic acid would have one carbon with excess C^{13} in it, and the butyric acid two such carbons. An example of the calculation of the C^{13} in the butyl and ethyl alcohols follows:

Toluene fraction:

Butyric acid = 5.30 mM;

Propionic acid = 0.12 mM;

Per cent excess C^{13} in carbon of fraction = 1.24.

Let X = per cent excess C^{13} in the one labeled carbon of the propionic acid and the two labeled carbons of the butyric acid. Then:

$$0.12X + (2 \times 5.30)X = (3 \times 0.12 + 4 \times 5.30)1.24;$$

$$X = 2.49.$$

$$\text{The } C^{13} \text{ of propionic acid} = \frac{2.49}{3} = 0.83. \text{ The } C^{13} \text{ of the butyric acid or butyl alcohol}$$

$$= \frac{2.49}{2} = 1.25.$$

Benzene fraction:

Propionic acid = 0.236 mM;

Acetic acid = 1.87 mM;

Per cent excess C^{13} in carbon of fraction = 0.66.

Let Y = excess C^{13} in the acetic acid:

$$(2 \times 1.87)Y + (3 \times 0.236)0.66 = (2 \times 1.87 + 3 \times 0.236)0.66.$$

$$Y = 0.63.$$

The composition of the alcohol mixture which was oxidized was 8.19 mM of butyl alcohol and 1.89 mM of ethyl alcohol. Therefore, 8.19×0.033 or 0.27 mM of acetic acid was formed from the butyl alcohol and 1.89 mM from ethyl alcohol. The 0.033 is the factor obtained by oxidation of pure butyl alcohol.

Let Z = per cent excess C^{13} in the acetic acid of ethyl alcohol origin.

$$(2 \times 0.27)1.25 + (2 \times 1.89)Z = (2 \times 0.27 + 2 \times 1.89)0.63.$$

$$Z = 0.54 \text{ per cent excess } C^{13} \text{ in ethyl alcohol.}$$

It is obvious that the determination of the C^{13} in the ethyl alcohol is not as reliable as for the other compounds. It is the best approximation possible under the conditions of these experiments.

Attention is called to three errors that have been noted during the development of these methods:

1. Stahly, *et al.* (31) reported that only acetic and butyric acids are formed in the oxidation of butyl alcohol by dichromate. In our determination, the acids from the oxidation of pure butyl alcohol were determined by the partition method of Osburn, *et al.* (25) and propionic acid was found to be a product. The presence of propionic acid was further established by using the distillation procedure of Schicktan, *et al.* (24) and partitioning the acids in the benzene distillate. The mixture was found to be 43 molar per cent acetic acid and 57 molar per cent propionic acid. There was no butyric acid in the fraction. The recovery of acetic acid in the benzene was in complete agreement with that determined on the unfractionated acids. These findings do not alter in any way the procedure of Stahly, *et al.* (31) since the partition constant for the oxidation product of butyl alcohol is the same as they reported. The fact is now revealed, however, that this constant does not represent a mixture of acetic and butyric acids but rather a mixture of acetic, propionic, and butyric acids.

2. It has been found that the partition constant for pure butyric acid is incorrect as given by Osburn, *et al.* (25). The correct values are:

$$K_1 = 9.7 \qquad K_2 = 54.5$$

Substituting these values the equations become:

$$\text{Acetic acid} = 4.356K_1 - 3.244K_2 + 134.53.$$

$$\text{Propionic acid} = -6.358K_1 + 8.990K_2 - 427.64.$$

$$\text{Butyric acid} = 2.002K_1 - 5.746K_2 + 393.74.$$

Apparently the butyric acid used by Osburn, *et al.* for determining the partition constants was not pure. When determination of the acids from butyl alcohol oxidation was made using the incorrect constants a negative value was found for propionic acid. Only when the new values for the butyric acid constants were substituted in the equation were reasonable values obtained.

3. Schicktan, *et al.* (24) state that only acetic and formic acids are recovered in the benzene distillate, but it has been our experience that part of the propionic acid passes into the benzene distillate. The separation of acetic and butyric acids is more satisfactory, and apparently in a mixture of acetic, propionic, and butyric acids, very little or none of the butyric acid is in the benzene distillate, while the propionic acid is distributed in both fractions.

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The Germicidal Action of Iodine

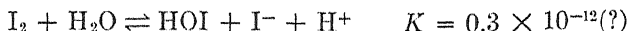
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INTRODUCTION

The quantitative aspects of the germicidal action of iodine are not easily determined because of the number of reactions which iodine undergoes in aqueous solutions. To be considered are the hydrolysis equilibrium,



the ionization of hypoiodous acid,

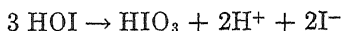


and perhalide formation,



The equilibrium constants for the hydrolysis equilibrium of iodine, (1, 2, 3) which appear in the literature are in disagreement but from the values given one would assume that in the hydrogen ion concentration range of biological experiments, iodine would occur both as I_2 and as HOI . Thus, in this aspect iodine differs from chlorine which exists as Cl_2 in aqueous solutions in appreciable amounts only below pH 2. On the other hand, the ionization of hypochlorous acid is a major factor in determining the killing action of chlorine solutions (4) but from the ionization constant of hypoiodous acid (5) it can be seen that HOI will be almost completely un-ionized throughout the biological pH range. In the presence of iodide ion, periodides are formed to the extent indicated by the equilibrium constant (6) and with an expected effect on the germicidal action. Higher periodides are also formed but in the dilute solutions used in our bacteriological experiments these would not be significant.

In addition to the equilibrium reactions, experiments with iodine are complicated by a decomposition reaction,



This could result in considerable loss in germicidal activity under conditions which favor this reaction since iodide and iodate are known to be inactive.

METHODS

The bacteriological methods are those described in our earlier studies with chlorine (4). Essentially they involve the determination of the time for 99% killing of *Bacillus meliens* spores in buffered distilled water which has been treated to render it free from oxidizing and reducing substances. Experiments in which there was doubt as to whether equilibrium had been attained were repeated with the indicated modifications to insure that the spores were added only to solutions at equilibrium.

In those experiments where loss of the active iodine occurred the iodine was added at zero time to a suspension of spores in the buffered water. Precautions were taken to minimize loss of iodine by volatilization. Iodine solutions were prepared without adding iodide by dissolving iodine crystals in alcohol and diluting in the test buffer solutions. Hypoiodous acid solutions free from I_2 were prepared by dissolving iodine crystals in water and shaking with excess HgO to remove the iodide, thus pulling the hydrolysis equilibrium completely to the right as shown in the first equation. In bacteriological experiments involving mercury, sulfide as well as sulfite was added to neutralize bacteriostatic action. For determining the hydrolysis constant, I_2 was estimated from a standard curve prepared with the Evelyn colorimeter at pH 3.5 where all the iodine exists in the molecular form. The total of I_2 plus HOI was determined by titration with arsenite in neutral solution. Titration in acid solution with thiosulfate determines the total oxidants which includes the iodate. All values given are in p.p.m. I_2 computed from the titration.

RESULTS

The values reported for the hydrolysis constant vary from 0.5×10^{-10} to 0.3×10^{-12} . Because HOI is colorless the value can be estimated by measuring the I_2 in solution colorimetrically at various pH levels. A series of buffers was prepared in the desired pH range, iodine was added, and the color measured immediately and after a few minutes to determine if equilibrium had been obtained or if decomposition was occurring. Equilibrium was instantaneous, and appreciable decomposition occurred only at the highest pH values. From the fraction of the added iodine present as I_2 the reported hydrolysis constant of the order of 0.3×10^{-12} was confirmed.

Our first studies on the effect of pH showed a marked decrease in activity at the higher pH values. Such a decrease in activity is due partly to the lesser activity of HOI as compared to I_2 but mainly due to the increased decomposition of the iodine to iodate when the HOI concentration is high. A study of this decomposition rate indicates that the amount and type of buffer used greatly influenced the formation

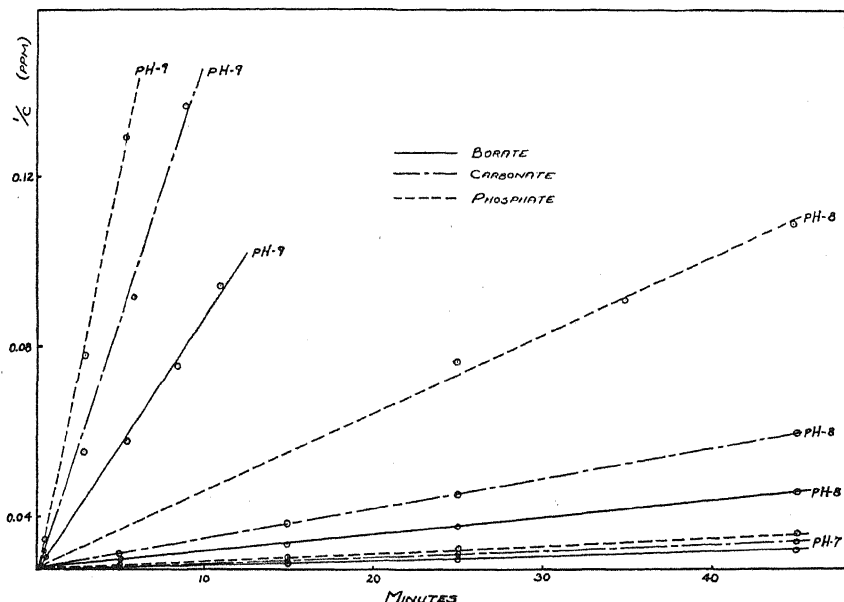


FIG. 1

Decomposition of Iodine Solutions to Iodate as Influenced by pH and Type of Buffer

of iodate from iodine solutions in the pH range where the concentration of HOI is significant. From Fig. 1 it is evident that when the reciprocals of the concentrations, as measured by the neutral arsenite titration, are plotted against time, straight lines result whose slopes give a measure of the rate of decomposition. At a buffer concentration of 0.02 M, there is little difference in the decomposition rates between phosphate, borate or carbonate buffer solutions of iodine at pH 7.0 but the differences in decomposition rates become increasingly significant as the pH is raised to 8 and to 9. The slopes of these lines as well as

those obtained when concentration and temperature effects were derived from similar plots are given in Table I. The type of buffer, its concentration, the pH, and the temperature greatly influence the decomposition rates. In these studies the acid thiosulfate titration remained nearly constant, indicating that the decomposition did not result in loss of oxidant but was truly a result of iodate formation.

TABLE I
Decomposition of Iodine as Measured by Arsenite Titration in Buffer Solutions at 25°C.

Slope of plot $1/c$ against time ($\times 10^3$).

	pH 7	8	9
Phosphate 0.02 <i>M</i>	0.23	1.6	18.6
Carbonate 0.02 <i>M</i>	0.19	0.7	11.9
Borate 0.02 <i>M</i>	0.16	0.4	5.3
Borate 0.004 <i>M</i>			2.5
0.02			5.3
0.1			16.8
0.02 (15°C.)			1.7

After consideration of the decomposition rates, buffers were chosen to give maximum stability, and a pH curve on the killing of iodine was made. Table II shows that the pH values above 7.5 decrease the killing action of iodine as the fraction of HOI in solution increases.

TABLE II
Effect of pH on Killing of B. metiens Spores by 40 p.p.m. I₂ Without Added KI at 25°C.

pH	Iodine at Equilibrium (as p.p.m. I ₂)		99% Killing Time Minutes
	I ₂	HOI	
6	38.3	1.7	2.3
7	35.0	5.0	2.3
7.5	31.4	8.6	2.2
8	26.8	13.2	2.8
8.5	18.8	21.2	4.5
9.0	11	29	11

However, if one takes into account the increased decomposition at the high pH values it is evident that the HOI must exert considerable killing though the activity is only a fraction of that of I₂. Experiments with pure HOI solutions at pH 6.0 prepared by the HgO method indicate this activity to be from $\frac{1}{3}$ to $\frac{1}{5}$ that of I₂ at equivalent concentration.

The effect of periodide formation is easily studied since the addition of iodide to iodine solutions decreases the HOI concentration and thus the decomposition to iodate.

Iodine solutions at 40 and 10 p.p.m. were prepared with the addition of KI in concentrations varying from 10 to 40,000 p.p.m. The time required for 99% killing as well as the composition of the solutions at equilibrium are recorded in Table III. It is evident that the killing

TABLE III

Killing of B. metiens Spores by Iodine in the Presence of KI at pH 7.0 and 25°C.

Dosage (p.p.m.)		At Equilibrium* (as p.p.m. I ₂)			99% Killing Time
I ₂	KI	I ₂	I ₃ ⁻	HOI	Minutes
40	40,000	0.2	39.8	—	220
40	4,000	2.3	37.7	—	39
40	400	15.1	24.9	—	9.5
40	40	32.0	7.5	0.5	5.1
40	10	36.8	1.5	1.7	4.3
10	3,168	0.7	9.3	—	96.0
10	1,584	1.3	8.7	—	57.0
10	792	2.3	7.7	—	41.0
10	396	3.7	6.3	—	30.5
10	198	5.4	4.6	—	20.5
40	0	35.0	—	5.0	4.0
10	0	7.6	—	2.4	14.5
3	0	1.8	—	1.2	45.0

* Computed from the equilibrium constant, 1.4×10^{-3} , and from the hydrolysis constant, 0.3×10^{-12} .

rate decreases with increase in KI in proportion to the decrease in I₂ present at equilibrium. Examination of the killing times for those combinations which give about 2.3 p.p.m. I₂ at equilibrium shows that the killing action exerted by the KI₃ under these conditions is negligible. This is further demonstrated by the plot of the log of the concentration of I₂ at equilibrium against the log of the time for 99% killing (Fig. 2). In the range of these concentrations all points fit reasonably well on a single straight line described by the equation

$$\log t = -.74 \log c + 1.92.$$

A similar plot with chlorine gives a straight line with a slope of about unity (7) which indicates that the activity of iodine does not change as rapidly with concentration changes as does the activity of chlorine.

The germicidal activity of iodine in the presence of excess KI should not vary with pH since under such circumstances the HOI concentration is negligible even at pH 9. The results of such an experiment are

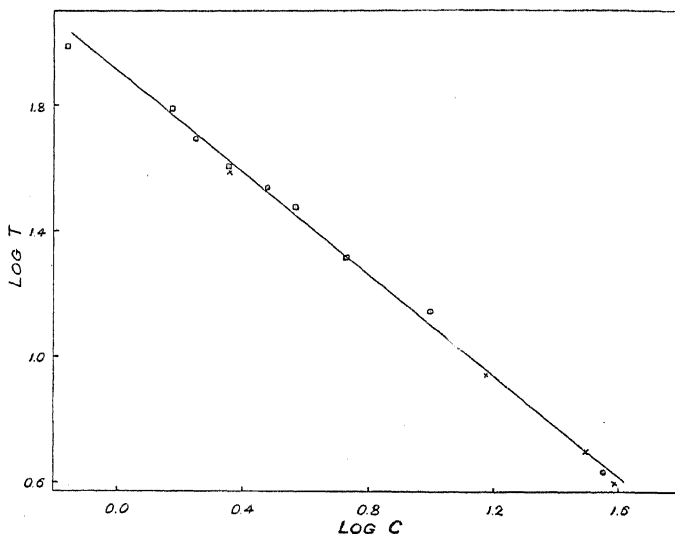


FIG. 2

Effect of Concentration of I_2 at Equilibrium on Killing Time

Crosses and squares represent the data involving 40 p.p.m. and 10 p.p.m. iodine dosage respectively from Table III. Circles represent data gathered in the absence of added KI.

shown in Table IV. Within the error of the experimental method, the killing action is equal for all pH values. These results are of fundamental importance not only in the study of iodine but also in our earlier studies with chlorine where wide changes in activity with pH occurred,

TABLE IV

*Effect of pH on Killing of *B. metiens* Spores by 40 p.p.m. I_2 in the Presence of 3,168 p.p.m. KI at 25°C.*

pH	I_2 at Equilibrium p.p.m.	99% Killing Time Minutes
6	2.8	34
7	2.8	36
8.1	2.8	38
9.2	2.8	37

since they show that the effect of pH is on the halogen and not on the organisms themselves.

Temperature experiments carried on in the presence of added KI show that lowering the temperature 10°C. results in about a fourfold increase in killing time (Table V). The I₂ concentration at equilibrium

TABLE V
*Effect of Temperature on the Killing of B. metiens Spores at pH 7.0
by 40 p.p.m. I₂ in the Presence of 400 p.p.m. KI*

Temperature	I ₂ at Equilibrium p.p.m.	99% Killing Time Minutes
35°C.	18	2.5
25°C.	15	9.3
15°C.	13	38.5
5°C.	11	175.0

varied with temperature, and the figures given are estimated from the equilibrium data of Dawson (8). Under such conditions iodine is very stable, and no loss occurs during an experiment extending over several hours. The effect of temperature on the germicidal action of iodine is considerably greater than on chlorine as Levine (7) has shown that the activity of chlorine increases less than twofold for each 10°C. rise in temperature.

Preliminary experiments indicate that iodine does not react in a manner analogous to chlorine in the presence of certain nitrogen compounds. The addition of equimolar concentrations of ammonia, succinimide, or *p*-toluenesulfonamide to iodine solutions at pH 7.0 does not change the rate of killing of *B. metiens* spores.

SUMMARY

The sporicidal action of iodine at pH values ordinarily encountered is due primarily to the I₂ although at high pH values HOI may also exert some action. KI₃ has no apparent activity but large amounts of KI must be added to iodine solutions to convert an appreciable fraction into this inactive form. Under certain conditions the decomposition of hypoiodous acid to iodate proceeds rapidly and introduces considerable error into experiments with iodine. The activity of iodine varies with temperature to a greater extent and with concentration to a lesser extent than does the activity of chlorine. A number of nitrogen compounds tested did not depress the germicidal activity of iodine.

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Preparation of Adenosine Triphosphate from Bovine Skeletal Muscle

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INTRODUCTION

Inasmuch as considerable work has been done on the preparation of adenosine triphosphate from the skeletal muscles of the rabbit and other small animals, it was decided to investigate the use of bovine skeletal muscle as a source. Kerr (3) in his description of a method for the isolation of adenosine triphosphate from rabbit muscle, gives a rather complete survey of the literature with regard to methods of isolation. He arrives at a method which is more or less a modification of the methods used by Fiske and Subbarow (2), Lohmann (4), and Barrenschenn and Filz (1). Needham (6) described a method considerably less complicated than that of Kerr. She, likewise, used rabbit skeletal muscle. Both Kerr and Needham obtained the dibarium salt of adenosine triphosphate.

It was decided to prepare the dibarium salt of adenosine triphosphate from bovine skeletal muscle by Kerr's procedure. Later Needham's method was applied and found to be satisfactory. The procedures will be described inasmuch as minor modifications were introduced in some cases.

EXPERIMENTAL

The Preparation of the Dibarium Salt by Kerr's Procedure

Twelve and seven-thenths kg. of "canner's beef" were obtained from the slaughter floor as soon as possible after the death of the animal. The muscle, free of fat, was ground in a meat chopper and placed in a 20-gallon crock containing a solution of 50 l. of water, 18 kg. of ice, and 4.5 kg. of trichloroacetic acid. The suspension was agitated for three hours by mechanical stirring, and then placed in the cooler over night. The next morning, the ice having melted, the suspension was filtered through a filter

bag. While still in the cooler, the filtrate was made neutral to phenolphthalein with 40% NaOH. The neutralized filtrate had a volume of about 73.6 liters. This filtrate was made up to 0.2% acetic acid by adding 148 ml. of glacial acetic acid; 3680 ml. of a 20% mercuric acetate solution were added to the filtrate (5 ml. of 20% mercuric acetate per 100 ml. of filtrate). A precipitate settled out immediately. The precipitated mixture was placed in the cooler over night.

The mercury precipitate was centrifuged off with the Sharples centrifuge, suspended in about 1 liter of water, and decomposed by passing hydrogen sulfide through the suspension for 1½ hours with constant stirring. During the decomposition the solution was kept in an ice bath. The mercury sulfide was centrifuged off and washed in a cup centrifuge. The combined washings and supernatant liquids were brought to a pH of about 8 with 40% NaOH. About 10 g. of sodium acetate were added, and hydrogen sulfide was bubbled through the solution for 10 minutes. During this time the iron sulfide precipitated. The suspension was filtered through a pad of Hyflo filter aid with suction. The filtrate was brought to a pH of 5-6, and aerated until paper moistened with lead acetate was no longer darkened when held over the solution during aeration. 318 ml. of 25% barium acetate solution were added (25 ml. of 25% barium acetate per kg. of muscle) to the aerated solution and then 6320 ml. denatured alcohol (2 volumes of alcohol) were added. The barium salt began to precipitate at once. It was allowed to settle in the cooler over night.

The barium salt was centrifuged off with the Sharples centrifuge. The precipitate was redissolved in 0.1 N HCl and precipitated with alcohol again. The precipitate was again centrifuged off on the Sharples centrifuge. The precipitate was dissolved in about one liter of 0.1 N HCl as before. (The precipitate is dissolved best by first suspending the precipitate in water and then adding enough 3 N HCl to make the solution 0.1 N with respect to HCl.) The barium salt was reprecipitated by adding 190 ml. of 25% barium acetate solution (15 ml. of 25% barium acetate per kg. of muscle), and then adding sufficient saturated Ba(OH)₂ to make the solution barely alkaline to phenolphthalein. This procedure was repeated twice, and then three times without the Ba(OH)₂ addition. For this and all subsequent manipulations of the precipitate, the cup centrifuge was used. The barium salt was washed three times with water, once with 70% alcohol, twice with 95% alcohol, once with ether. The material was air dried and then placed in a vacuum desiccator over H₂SO₄ and KOH. The barium salt weighed 14.5 grams.

Analysis. C₁₀H₁₂O₁₃N₅P₃Ba₂ (777.88), dried at 100°C., in vacuum over P₂O₅.

Calculated	N 9.00,	P 11.97.
Found	8.52,	11.72.

The Preparation of the Dibarium Salt by Needham's Procedure

Eleven and eight-tenths kg. of "canner's beef," freshly obtained from the slaughter floor, were quickly frozen with dry ice and ground with a hammer mill using a rather coarse screen. The frozen muscle together with twelve liters of 10% trichloroacetic acid was placed in a 20-gallon crock. When the mixture had thawed, it was agitated for several hours at room temperature with a mechanical stirrer. The extraction mixture stood over night in a 5°C. cooler. Next morning, the mixture was filtered through a muslin bag and subsequently through large filter paper. The residue was

re-extracted for about 15 minutes with an equal volume (12 liters) of 4% trichloroacetic acid. This extract was filtered as before.

The combined extracts were brought to pH 6.8 with 40% NaOH. A solution of 50% barium acetate was added until no further precipitation occurred. After the precipitate had settled, the supernatant was siphoned off and discarded. After centrifugation, the precipitate was suspended in 0.2 *N* HNO₃ until faintly blue to Congo red paper. The insoluble material was centrifuged off and discarded. The supernatant was brought to 0°C. in an ice-salt bath, and 50 ml. (3–5 ml. for each kg. of muscle) of Lohmann's reagent (100 g. HgNO₃·8H₂O dissolved in a mixture of 25 ml. HNO₃, sp. gr. 1.4, and 25 ml. water) were added, thus precipitating the mercury salt of adenosine triphosphate. The supernatant was tested for complete precipitation by adding an additional quantity of reagent. After standing for 15 minutes in the cold the mercury salt precipitate was centrifuged off. The mercury salt was suspended in a minimum of water and decomposed by passing H₂S through the suspension with agitation. The material was kept in an ice bath during this operation. After the mercury salt had been decomposed, the HgS was centrifuged off and the supernatant was aerated until lead acetate paper was no longer colored when held over the container.

The pH of the aerated solution was brought to 6.8 by adding dilute NaOH solution. A precipitate began to form when pH 6.8 was approached because of the presence of a small amount of barium ion in the solution. A solution of 25% barium acetate was added until no further precipitation occurred. The precipitate was allowed to settle over night in 5°C. chill room. In the morning, the precipitate was centrifuged off, washed with 1% barium acetate, 50% alcohol, 75% alcohol, 95% alcohol, and finally with ether. The material was then placed in a vacuum desiccator to dry over P₂O₅. Later the material was dried at 100°C. in vacuum over P₂O₅ for one hour. The dried salt weighed 18.46 grams. Analytical results showed this preparation to be rather impure. In order to purify it further, the barium precipitation was repeated. The salt was dissolved in 0.2 *N* HNO₃ and reprecipitation occurred when the pH was adjusted to 6.8 by adding dilute NaOH. Completeness of precipitation was ascertained by testing the supernatant with the addition of 25% barium acetate solution. The precipitate was centrifuged, washed well with 1% barium acetate solution, 50% alcohol, 75% alcohol, 95% alcohol, and with ether. The salt was first dried in a vacuum desiccator and then in vacuum at 100°C. over P₂O₅. The weight of the dried purified salt was 14.64 grams.

Analysis. C₁₀H₁₂O₁₃N₆P₃Ba₂ (777.88), dried at 100°C., in vacuum over P₂O₅.

Calculated	N 9.00,	P 11.97.
Found	8.73,	12.09.

Twelve g. of barium adenosine triphosphate obtained from beef muscle by Kerr's procedure were converted by his procedure to adenylic acid. Kerr's procedure for adenylic acid was modified to the extent that the hydrolysis with Ba(OH)₂ was carried out by boiling slowly over a gas flame under a reflux condenser. The saturated Ba(OH)₂ was added during the hydrolysis over a period of 30 minutes until the pink color due to phenolphthalein persisted. A yield of 4.08 g. adenylic acid was obtained in the form of clusters of crystalline needles with a m.p. 191–192, uncorrected.

Analysis. $C_{10}H_{14}N_6O_7P$ (347.27).

Calculated	N 20.17,	P 8.93.
Found	19.60,	9.06.

All analyses reported in this paper were made by Dr. T. S. Ma, University of Chicago.

SUMMARY

Barium adenosine triphosphate has been prepared from beef muscle by two methods. Needham's method is less complicated than Kerr's procedure, and yielded a satisfactory product.

Barium adenosine triphosphate from beef muscle was converted to adenylic acid by hydrolysis with $Ba(OH)_2$.

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Elimination of Error in the Volhard Analysis for Chloride by Titration in Acetic Acid

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INTRODUCTION

The Volhard method (1) for the volumetric determination of chloride has the defects that adsorption of silver ions on the precipitate tends to make the results high and that re-solution of the silver chloride in the presence of the less soluble silver thiocyanate tends to make them low. Several modifications have been proposed to circumvent these difficulties: Ion exchange can be prevented by filtering off the silver chloride before titrating the excess silver, but according to Kolthoff (2) the results are about 0.7 per cent high owing to adsorption of silver ions. Ether, *sec.* butyl alcohol, or other similar liquid may be added to the solution to form a second liquid phase, which will coagulate the silver chloride at the interface. This slows down but does not prevent ion exchange. The adsorbed ions can be removed by prolonged shaking, after the first appearance of an end point, but then opportunity for ion exchange is afforded.

It has now been found that errors from both of these sources are eliminated if the titration is made in 40–65 per cent acetic acid solution. Under these conditions the end point shows no fading in 24 hours, and hence silver chloride is presumably rendered less soluble than silver thiocyanate. Chloroform may be added to produce a second liquid phase in order to facilitate coagulation of the silver chloride. Chloroform is much superior to ether or other light liquids in that it coagulates the silver chloride below the aqueous layer instead of above it and hence makes observation of the end point in the supernatant layer easier.

EXPERIMENTAL

In establishing the worth of these modifications the procedure was as follows: The acetic acid, water, chloroform, nitric acid, and a known amount of 0.0114 *N* HCl were put into 1 × 8 inch glass-stoppered test tubes and shaken briefly to produce equilibrium. A known amount of 0.02 *N* AgNO₃ was then added, and the tubes were vigorously shaken for one minute to coagulate the silver chloride. Ferric alum indicator was then added and the excess silver titrated with 0.02 *N* potassium thio-

TABLE I
Effect of Acetic Acid Concentration on the Estimation of Chloride

H ₂ O ml.	Acetic Acid added ml.	Acetic Acid in aqueous layer Vol. per cent	KSCN used ml.	Chloride found mM	Error per cent
10	20	43	9.760	0*	
30	0	0	4.480	0.1078	-3.2
30	0	0	5.000	0.0974	-12.6
20	10	22	4.380	0.1098	-1.4
20	10	22	4.390	0.1096	-1.6
15	15	33	4.330	0.1108	-0.5
15	15	33	4.300	0.1114	0
10	20	43	4.320	0.1110	-0.4
10	20	43	4.300	0.1114	0
10	20	43	4.300	0.1114	0
5	25	54	4.290	0.1116	+0.2
5	25	54	4.310	0.1112	-0.2
0	30	64	4.300	0.1114	0
0	30	64	4.310	0.1112	-0.2

* No chloride present. All other test tubes contained 0.1114 mM HCl. All test tubes contained 9.950 ml. of 0.02 *N* AgNO₃, 15 ml. chloroform, 0.5 ml. saturated ferric alum solution, and 1 ml. HNO₃ (except the last two which contained 2 ml.).

cyanate. The bulk of the thiocyanate was added first and the tube shaken vigorously for one minute. The remaining thiocyanate was added in increments of 0.01 ml. and the tube gently tilted to distribute the solution after each addition. After the first indication of an end point, the tube was shaken vigorously for about half a minute and again after each subsequent addition of thiocyanate until a permanent color change was obtained. The final end point was determined by comparing the color with that of a similar tube containing the same amount of silver solution, but no chloride, to which thiocyanate had been added until an easily discernible color change was produced. The titer of this tube also served to standardize the thiocyanate solution. A recognizable color difference is produced by the addition of a few thousandths of a milliliter of thiocyanate to 50 ml. of solution when looking through the depth of the tube.

That acetic acid does not interfere with the end point was shown by titrating 9.950 ml. of 0.02 *N* AgNO₃ in 30 ml. water plus 20 ml. acetic acid with 0.02 *N* KSCN solution. The titers were 9.792 and 9.788 ml. In the absence of acetic acid titers of 9.790, 9.788 ml. were obtained.

TABLE II

Determination of Chloride in HCl, Urine, Mixtures of HCl and Urine, Blood Filtrates, and Mixtures of Blood Filtrates and HCl

		Chlorides		
HCl ml.	Urine or Blood Filtrate ml.	Found mM	Calc. mM	Difference mM
(a) Urine				
1.004	0	0.1021 ¹		
1.004	0	0.1019		
1.004	0	0.1026		
—	—	(0.1022) ⁷	0.1024	-0.0002
0	0.484 ³	0.1226		
0	0.484	0.1228		
0	0.484	0.1230		
0	0.484	0.1230		
—	—	(0.1229) ⁷	—	—
0.484	0.484	0.1719		
0.484	0.484	0.1717		
0.484	0.484	0.1721		
—	—	(0.1719) ⁷	0.1722	-0.0003
1.004	0	0.1006 ²		
1.004	0	0.1002		
—	—	(0.1004) ⁷	0.1004	0
0	0.484 ⁴	0.1253		
0	0.484	0.1255		
—	—	(0.1254) ⁷	—	—
0.484	0.484	0.1740		
0.484	0.484	0.1739		
0.484	0.484	0.1743		
—	—	(0.1741) ⁷	0.1738	+0.0003
(b) Blood Filtrate				
1.004	0	0.1006		
1.004	0	0.1002		
—	—	(0.1004) ⁷	0.1004	0
0	4.996 ⁵	0.0402		
0	4.996	0.0404		
0	4.996	0.0400		
—	—	(0.0402) ⁷	—	—
1.004	4.996	0.1410		
1.004	4.996	0.1406		
—	—	(0.1408) ⁷	0.1406	+0.002
0	4.996 ⁶	0.0400		
0	4.996	0.0402		
0	4.996	0.0399		
—	—	(0.0400) ⁷	—	—
1.004	4.996	0.1402		
1.004	4.996	0.1407		
—	—	(0.1405) ⁷	0.1404	+0.0001

¹ This and the 2 values that follow are for 0.1020 N HCl.

² This and the value that follows are for 0.1000 N HCl.

³ Urine sample No. 1.

⁴ Urine sample No. 2.

⁵ Blood filtrate from tungstic acid.

⁶ Blood filtrate from cadmium hydroxide.

⁷ Average values.

A series of experiments, the data from which are given in Table I, showed that correct results are obtained when the aqueous layer contained 40–65 per cent acetic acid (assuming for convenience that the lower phase is pure chloroform). The end point was stable when the acetic acid concentration was only 22 per cent but the chloride values were low. In the absence of acetic acid the end point faded in about an hour and the results were very variable. The color of the end point in the presence of acetic acid is orange rather than rose as it is in aqueous solution.

Since Volhard's method or modifications thereof are frequently used for the measurement of chloride in urine (3) and in blood (4), the procedure described above was applied to 2 samples of urine, to blood filtrates, obtained after precipitating the protein with tungstic acid and with cadmium hydroxide, and to mixtures of these materials with known amounts of HCl. The results, given in Table II, show satisfactory agreement between the calculated and measured values.

If the urine is so deeply colored as to interfere with the matching of colors at the end point, the color in the tube containing the blank may be brought to that of the urine by adding a few drops of brom-phenol blue indicator. It was found that with blood filtrates from tungstic acid the silver chloride coagulated quite slowly, but with filtrates from cadmium hydroxide coagulation was as rapid as when titrating solutions of HCl.

SUMMARY

1. The errors in the Volhard method for estimating chloride caused by ion-exchange and by occlusion, have been eliminated by conducting the titration in a medium containing 40 to 65 per cent acetic acid.
2. The modified procedure is applicable to urine and to blood filtrates.

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A Note on the Amino Acids Yielded by Yeast, Sunflower Seed Meal, and Sesame Seed after Hydrolysis of the Fat Free Tissue

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INTRODUCTION AND EXPERIMENTAL

The current interest in plant sources rich in protein as substitutes for animal protein in nutrition, is brought out by the publication of a symposium on this subject (1). "The idea of the use of yeast as a source of food protein for man and the higher animals is not a new one; it has, however, been given renewed emphasis by the exigencies of the food situation during the war. Yeast is a highly nitrogenous by-product of the fermentation industries which heretofore has been largely wasted. It also presents the possibility of a synthetic production of protein from exceptionally simple compounds independent of seasons and crops" (Osborne and Mendel, 2).

The yeast used for this analysis was a debittered brewer's yeast, prepared for human consumption by the Harris Laboratories. Besides the brewer's yeast, a sample of solvent extracted sunflower seed meal obtained from the Viobin Corporation and whole, ground, sesame seed flour furnished through the kindness of Dr. H. J. Almquist were analyzed.

Before amino acid analysis, the plant products were thoroughly extracted with hot benzene and ether to remove fatty substances, and then dried at 100°C. The following methods were used: nitrogen by the micro-Kjeldahl-method; arginine, histidine, and lysine by a small scale modification of the Kossel-Kutscher method following hydrolysis with 8 *N* H₂SO₄ (3); tyrosine and tryptophan by Lugg's adaptation of the Millon reaction using 5 *N* NaOH for hydrolysis (*cf.* 3); phenylalanine by a modification of the Kapeller-Adler procedure (3) with either 5 *N* NaOH or 7 *N* H₂SO₄ for hydrolysis; cystine by the Folin or Fleming-Vassel methods following hydrolysis with a 1:1

mixture of 20 per cent HCl and 90 per cent HCOOH (*cf.* 3); methionine by the McCarthy-Sullivan method after 6 *N* HCl hydrolysis (*cf.* 3); threonine by oxidation to acetaldehyde after 6 *N* HCl hydrolysis (3); leucine, isoleucine, and valine by an adaptation of the microbiological methods of Shankman (4) and of McMahan and Snell (5) using 3 *N* HCl hydrolysis at 15 lbs. pressure; and glycine by the Zimmer-

TABLE I

Comparative Amino Acid Analyses of Yeast, Sunflower Seed, Sesame Seed, Chopped Beef, and Casein Proteins

(Calculated to 16.0 per cent of Nitrogen)

	Yeast (Harris) per cent	Sunflower seed per cent	Sesame seed per cent	Chopped beef per cent	Casein per cent
Arginine	3.1	8.2	8.7	7.7	4.1
Histidine	3.3	1.7	1.5	2.9	2.5
Lysine	7.1	3.8	2.8	7.2	7.5
Tyrosine	3.8	2.6	3.5	3.4	6.4
Tryptophan	1.2	1.3	1.8	1.3	1.2
Phenylalanine	4.5	5.7	8.3	4.9	5.2
Cystine	1.1*; 1.2	1.4*; 1.6	1.3*	1.3	0.4
Methionine	2.7	3.4	3.1	3.3	3.5
Threonine	5.5	4.0	3.6	5.4	3.9
Leucine	7.3	6.2	7.5	7.7 ^a	12.1
Isoleucine	6.0	5.2	4.8	3.0	6.5
Valine	5.3	5.2	5.1	3.5 ^a	7.0
Glycine			9.3		

* Fleming-Vassel

^a Schweigert, *et al.* (9) find 7.7 per cent of leucine and 5.0 per cent of valine by essentially the same procedure. The differences in valine may be due to the samples analyzed. The determinations of leucine, isoleucine, and valine were carried out on the same preparation by the chemical method in 1940; the results indicated: leucine 11 per cent, isoleucine 3.0 per cent, and valine 3.6 per cent (3). Recent microbiological analyses on previously analyzed samples show that our values for leucine, by the chemical method, were often too high.

mann-Patton *o*-phthaldialdehyde reaction following hydrolysis with 6 *N* HCl (3). All values are calculated to 16.0 per cent of nitrogen ($N \times 6.25$). No account is taken of the non-protein nitrogenous products which in the case of yeast, at least, may amount to over 15 per cent of the true protein (1, 7).

The *in vitro* digestibility of the yeast and sunflower seed proteins were compared to that of dried skim milk proteins, using suboptimal quantities of commercial pancreatin at pH 8.0 to 8.5 at 37°C. Formal titration indicated that these three proteins were digested at approximately the same rates and reached about the same final values in 5 days' digestion.

RESULTS

The amino acid values are given in Table I. For the sake of comparison, an analysis of a sample of chopped beef, and values for casein, taken from data in (3), are also given.

The controversial literature on the nutritive value of yeast proteins (1, 2, 6, 7) may be due to variations in the amino acid composition of the yeasts studied, or to difficulties in digestibility (*cf.* 1 for a discussion of the effects of digestibility on nutritive value). The yeast used in this analysis appears to contain amino acids in balanced proportion and to be well digested by pancreatin.

Sunflower seed proteins are lower in lysine, but richer in cystine plus methionine than yeast proteins. The tryptophan value in sesame seed proteins is significantly higher than the other proteins and is similar to the value in linseed meal (8).

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The Antagonism by Spermine and Spermidine of the Antibacterial Action of Quinacrine and Other Drugs

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INTRODUCTION

Silverman and Evans (1) have described the antagonism of the antibacterial action of Atabrine¹ by spermine and spermidine when *Escherichia coli* was used as the test organism, and the appearance of visible turbidity in a bacto-peptone medium was taken as an endpoint. We have studied the antagonism of these amines against the antibacterial action of Atabrine and other drugs, using as an endpoint the turbidity developed during the time required for control cultures to attain maximal growth. Media containing peptone are known frequently to exert effects antagonistic to the anti-bacterial action of many drugs (2, 3, 4, 5). Accordingly, our tests were carried out using not only a bacto-peptone medium but also a medium containing salts, glucose and asparagine.

EXPERIMENTAL

Methods

The media used in these tests had the following compositions:

Salt-glucose-asparagine medium		Bacto-peptone medium	
NH ₄ NO ₃	5 g.	Bacto-peptone	10 g.
Na ₂ SO ₄	5 g.	NaCl	6 g.
K ₂ HPO ₄	4 g.	Glucose (anhydrous)	2 g.
MgSO ₄ (anhydrous)	0.2 g.	K ₂ HPO ₄	1 g.
Asparagine	2 g.	Distilled water to make	800 ml.
Glucose (anhydrous)	10 g.		
Distilled water to make	800 ml.		

¹ Winthrop Chemical Co. has applied its trademark 'Atabrine' to quinacrine.

The media were put up in 4 ml. amounts in pyrex tubes and the test substance or water was added to make a final volume of 5 ml. Sterilization was effected by autoclaving at 115°C. (10 pounds) for 5 minutes. In some cases test substances were sterilized separately, either by autoclaving or by filtering through fritted glass, and were added to the tubes aseptically. Atabrine and Mapharsen² solutions were never autoclaved. All glassware was scrupulously cleaned chemically before use.

The organism employed was a laboratory strain of *Escherichia coli*. Two strains were carried from the stock culture: one in the salt medium and one in bacto-peptone medium. Transfers of these strains were made daily. The incubation temperature was 37°C. It should be noted that in the salt medium this organism did not remain viable longer than 36 hours. In the experiments using the salt medium, the inoculum consisted of 0.1 ml. of a 1:10,000 dilution, in distilled water, of a culture grown for 24 hours in the same medium. Studies of growth curves in this medium, using such an inoculum, showed that the peak of the growth curve was reached in from 17 to 20 hours, as determined by turbidity readings on a Klett-Summerson photoelectric colorimeter. For experiments using bacto-peptone medium, the inoculum used was 0.1 ml. of a 1:1000 dilution of a 24 hour culture in bacto-peptone. Here the peak of the growth curve was attained in from 6 to 8 hours. Control tubes were set up with each test and were checked frequently in order to determine when maximal turbidity had been attained. Growth was then stopped by keeping all tubes at 4°C. for at least 20 min. before turbidities were measured. Appropriate color blanks were run concurrently with each test. Control growth tubes were used in sufficient number to determine the normal variation in their turbidity. Variation in turbidity in these tubes was slight, and was less noticeable in the bacto-peptone medium than in the salt medium.

The spermine and spermidine were isolated from pancreas by Dr. D. K. Bosshardt, of these laboratories; Atabrine and the compound corresponding to the Atabrine side chain (diethylamino-amino-pentane) were kindly provided by the Winthrop Chemical Co.; propamidine, putrescine, quinine, Mapharsen, sulfanilamide, and sulfathiazole were obtained from commercial sources.

RESULTS

Atabrine Experiments. Concentrations of Atabrine between $M/5000$ and $M/10,000$ in salt medium completely inhibited the growth of the strain of *E. coli* employed. None of the amines tested was able to antagonize completely the bacteriostatic effect of these concentrations of Atabrine, but in the presence of a concentration of Atabrine ($M/10,000$) which barely allowed measurable growth to occur, 5 molar equivalents (m.e.) of spermine allowed approximately 75 per cent of normal growth. Five m.e. of spermidine permitted about 50 per cent of normal growth; a similar effect was produced by

² Parke, Davis and Co. has applied its trademark 'Mapharsen' to *m*-amino-*p*-hydroxy-phenyl arsenoxide hydrochloride.

2 m.e. of spermine. The amines themselves had a slight stimulatory effect on the rate of growth of this organism in the salt medium; such stimulation, however, was too small to account for the results just mentioned. Putrescine, a shorter chain diamine related to spermine and spermidine, exerted no antagonistic effect on Atabrine inhibition. Like the higher homologues it stimulated slightly the growth rate of the organism.

In the bacto-peptone medium complete bacteriostasis was produced by $M/2000$ Atabrine, but tubes containing $M/4000$ Atabrine were slightly turbid at the end of the test period. In a concentration of $M/2000$, spermine slightly inhibited growth in this medium, an effect not noted with spermidine or putrescine in the same concentration. Spermine in a concentration which did not inhibit growth ($M/10,000$) was used in the tests to be described.

Although the amines studied did not antagonize completely the bacteriostatic action of Atabrine on *E. coli* in the bacto-peptone medium, the following results were noted: 1.0 m.e. of spermine ($M/10,000$) in the presence of 2.5 m.e. of Atabrine allowed 50 per cent of normal growth; similar growth occurred with a molar ratio of spermidine to Atabrine of 2:1. Here, too, as in the salt medium, putrescine was ineffective as an antagonist.

In an exact repetition of the experiment described by Silverman and Evans (1), in which the time of first appearance of turbidity was used as an endpoint, we found that tubes containing $M/2000$ Atabrine completely inhibited growth during an incubation period of 96 hours, while tubes containing $M/2000$ spermine or spermidine in addition to $M/2000$ Atabrine showed visible growth in 5 to 6 hours, and control tubes had a similar turbidity in 4 hours.

It appeared possible that Atabrine might owe its bacteriostatic activity either to the fact that it competed with spermine and spermidine for certain metabolic pathways by virtue of its side chain (the structure of which bears a chemical resemblance to that of these amines), or that something related to diethylamino-amino-pentane (the side chain of Atabrine) might itself be a normal metabolite which is partially replaceable by spermine or spermidine. However, in neither salt nor bacto-peptone medium did this compound (diethylamino-amino-pentane) antagonize the bacteriostatic action of Atabrine. Furthermore, unlike spermine, it did not inhibit the growth of

the organism in bacto-peptone medium in concentrations of $M/2000$ or $M/1000$.

Propamidine Experiments. In salt medium normal growth occurred in tubes having a spermine:propamidine molar ratio of 10:1, and a ratio of 1:1 was required to reduce growth to 50 per cent of normal. In bacto-peptone medium a ratio of 2.5:1 reduced growth to 50 per cent of normal. The spermidine:propamidine molar ratio required to reduce the growth rate to 50 per cent of normal was 10:1 in the bacto-peptone medium.

When the appearance of visible turbidity was used as an endpoint, it was found that $M/20,000$ propamidine prevented the appearance of growth of this organism for 6-7 hours after seeding, but the addition of $M/2000$ or $M/4000$ spermine to the $M/20,000$ propamidine allowed turbidity to appear in 4.5 hours, while growth in the control tubes was observed in 4 hours. Tubes containing $M/10,000$ propamidine were still clear at the end of 21 hours of incubation, while similar tubes containing $M/2000$ spermine showed growth in about 8 hours.

Miscellaneous Experiments. Neither spermine nor spermidine, in salt medium, had any antagonistic effect on the inhibition of growth of *E. coli* caused by sulfanilamide or sulfathiazole, except for that which could be accounted for by the acceleration of growth caused by these amines. Mapharsen apparently reacted chemically with spermine, for a brown color developed on incubation of tubes containing the two compounds. Antagonism of Mapharsen inhibition was noted in such tubes. A complete reversal of quinine bacteriostasis by spermine in the salt medium was noted when the molar ratio, spermine:quinine, was 1:2. A molar ratio of 1:3 (spermine:quinine) allowed 50 per cent of normal growth. In bacto-peptone medium the ratio for 50 per cent of growth was about 1:20.

DISCUSSION

Molar ratios were calculated from the amount of drug (inhibitor) which barely allowed growth of *Escherichia coli* and the amount of amine (antagonist) which, in the presence of the inhibitor, allowed 50 per cent of normal turbidity to develop at the end of the test period. As a comparison, it was determined that, in the salt-glucose-asparagine medium, 1 molar equivalent of *p*-aminobenzoic acid (PAB) antagonized approximately 300 molar equivalents of sulfanilamide. Using such a ratio (1:300) as a criterion it must be concluded that the spermine-spermidine antagonisms for the drugs tested here are of a relatively low order. Because these amines antagonize growth inhibitions of drugs not closely related in chemical structure, we may

assume that the antagonism is not entirely a specific one and that the amines tested apparently are not essential metabolites for the growth of *E. coli* under these conditions. The antibacteriostatic effects of peptone probably are also largely non-specific and are due to the improvement this medium brings about in the nutrient environment of the organisms (6).

We feel it is important to mention some of the bacteriological aspects influencing this type of work. The previous history and daily treatment of an organism used in such tests has considerable influence on the amount of any drug necessary to inhibit the growth of that organism. We feel, therefore, that antagonists:inhibitor molar ratios

TABLE I

Antagonist: Inhibitor Molar Ratios Calculated for 50% Normal Turbidity of Escherichia coli

Inhibitor antagonist	Medium	Ratio for 50% normal growth A/I*	Maximum antagonism observed % Normal growth	Ratio A/I
Atabrine Spermine	S†	2:1	75	5:1
Atabrine Spermine	BP‡	1:25	50	
Atabrine Spermidine	S	5:1	50	
Atabrine Spermidine	BP	2:1	50	
Propamidine Spermine	S	8:1	100	10:1
Propamidine Spermine	BP	2.5:1	60	10:1
Propamidine Spermidine	BP	10:1		
Quinine Spermine	S	1:3	100	1:2
Quinine Spermine	BP	1:20	90	1:12

* A = antagonist; I = inhibitor

† S = salt-glucose-asparagine medium

‡ BP = Bacto-peptone medium

indicate more accurately the results obtained than do mere statements of concentrations. However, these ratios also have been observed to shift, not only with the culture medium employed, the age and method of handling of the organism, and the concentration of inhibitor that must be employed, but even with the molar concentration of the antagonist.³ This was seen particularly in the case of PAB:sulfonamide ratios and the spermine:quinine ratios calculated from the bacto-peptone medium studies.

³ These observations agree with those of Dr. R. J. Strawinski (unpublished data).

SUMMARY

1. Spermine and spermidine showed some antagonism to the inhibitory effects of Atabrine, propamidine, and quinine on the growth of *Escherichia coli* in a bacto-peptone medium and in a salt-glucose-asparagine medium.

2. In all cases spermine showed greater antagonistic powers than did spermidine.

3. Complete reversal by spermine or spermidine of drug inhibition was never observed in the case of Atabrine. Such reversal was observed in the salt medium for both propamidine and quinine.

4. Inhibition by sulfanilamide and sulfathiazole of the growth of *E. coli* was not antagonized by either spermine or spermidine.

5. Diethylamino-amino-pentane (Atabrine side chain) exhibited little effect on the growth of *E. coli* and was unable to antagonize Atabrine inhibition.

6. Putrescine, an amine related to spermine and spermidine, was not active as an antagonist.

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The Methionine Content of Feedstuff Proteins

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INTRODUCTION

The elucidation of the role of methionine in the nutrition of the chick (1, 2) and the practical problem of the suboptimum methionine content of soybean oil meal (3, 4) has made desirable a knowledge of the methionine content of feedstuff proteins. Methionine analyses of a large number of isolated protein fractions from both animal and plant sources have been made (5-9), but the only data from determinations on feedstuff proteins as naturally found and consumed appear to be those reported by Block and Bolling (10).

The chemical estimations reported here were carried out according to the method of McCarthy and Sullivan (11), in which the methionine present in a hydrolyzate is allowed to react with nitroprusside under appropriate conditions and the red color produced is compared with standard solutions of methionine treated in an identical manner.

Biological estimations of methionine were made by utilizing the fact that raw soybean protein may be made to support good chick growth when supplemented with methionine or potent sources of this amino acid (3, 4). The biological estimations were intended to serve as a check on the chemical estimations. It seemed especially desirable to ascertain whether further examples might exist similar to the anomaly in raw soybean protein in which methionine is present but apparently unavailable to the animal.

A. CHEMICAL ESTIMATIONS

The samples were finely ground and the total crude protein ($N \times 6.25$) was determined by the Kjeldahl method. The amount of sample taken for hydrolysis varied from 0.4 g. to 5.0 g. depending on the methionine

content; in general, a sample was chosen which contained 5 to 15 mg. of methionine.

Two methods of hydrolysis were used: (1) heating in an oil bath at 125°–135°C. with 5–15 ml. 6 *N* HCl for 4–6 hours, or (2) digesting with pepsin in 0.1 *N* HCl. Neither of these methods effects complete hydrolysis of the protein, but, as McCarthy and Sullivan have shown (11), the same values are obtained after only enough hydrolysis to prevent protein precipitation during color formation as after complete hydrolysis. After the acid hydrolysis, 20 ml. water and 50–100 mg. of water-washed Nuchar were added, the mixture was boiled 20 minutes, filtered while hot through Whatman No. 50 paper with the aid of suction, washed repeatedly with 1 ml. portions of hot 0.1 *N* HCl followed by hot water, brought to pH 3 with 14 *N* NaOH, and diluted to 50 ml.

The pepsin solution was prepared immediately before use, and contained 30 mg. pepsin (Wilson Laboratories, 1 : 10,000) per 100 ml. of 0.1 *N* HCl. 35 ml. of this solution were added to the weighed sample and the mixture was placed in an air bath at 37°C. After 2 days, during which time the mixture was shaken occasionally, approximately 1.5 g. Celite filter aid (Johns-Mansville) were added and the mixture was filtered by suction (Whatman No. 50 paper). The residue was washed repeatedly with hot water, and the filtrate was made up to 50 ml. A drop of toluene was added to all hydrolyzates as a preservative.

Each feedstuff was hydrolyzed by pepsin. Other samples of most products were hydrolyzed by the acid method to give a comparison of the two methods. The proteins of some samples (*e.g.*, whole cereals) which were not conveniently treated by the acid method were brought into solution easily by pepsin. In most cases the solubility of the proteins in the presence of pepsin was very high, and although micro-Kjeldahl protein determinations were made on the hydrolyzates to check the amount of soluble protein when only the enzymatic method was employed, only small corrections were needed. Exceptions to this were alfalfa meal (65% of total protein dissolved), cottonseed meal (80%) and wheat bran (84%). With these substances, the methionine content could be expressed only as per cent of soluble crude protein, and it was necessary to assume that the soluble protein is representative of the total protein of the sample. Since there was no significant difference between analyses made by the acid and enzyme hydrolysis, the various results were averaged to give the values of Table I.

TABLE I

The Methionine Content of Various Feedstuff Proteins

Protein source	Crude protein (N × 6.25)	Method of hy- drolysis	Number of deter- minations	Methionine present in crude protein	Values from the literature (10)
	<i>per cent</i>			<i>per cent</i>	<i>per cent</i>
Animal products					
Blood fibrin, beef (Difco)	91.8	A, E†	4	3.1	2.6
Blood fibrin, beef (Swift)	91.1	A, E	3	2.9	
Blood serum, beef (Swift)	74.6	A, E	4	1.7	2.1
Casein	87.8	A, E	4	3.6	
Fishmeal, carp	58.6	A, E	6	2.7	
Fishmeal, sardine	69.6	A, E	5	3.1	
Fishmeal, shark	64.3	A, E	3	1.4	
Fishmeal, tuna	63.2	A, E	5	3.0	
Fish press water, menhaden, dried	60.8	E	4	0.9	
Fish press water, sardine, dried	66.5	E	3	1.9	
Gelatin	95.1	A, E		0.4	
Liver, beef, dried	66.6	E	4	2.7	
Meat scrap	67.3	A, E	4	2.0	3
Whey	11.0	E	4	2.1	
Plant products					
Alfalfa meal	18.5	E	4	2.3§	2.3
Barley	10.4	E	5	2.1	
Corn gluten meal	45.6	A, E	7	2.3	
Cottonseed meal	43.9	E	4	2.1§	1.6
Fermentation residue (butyl)	26.4	E	2	3.7	
Linseed meal	35.1	E	5	2.3	3
Peanut meal	42.7	E	2	1.7	0.9
Sesame meal	47.8	E	5	3.4	
Soybeans, raw	37.7	A, E	5	2.0	2.0
Soybean meal, solvent process	46.0	A, E	6	2.2	
Sunflower seed meal	46.7	E	2	3.9	3.4
Wheat	10.8	E	3	2.0	3
Wheat protein, hydrolyzed, commercial	48.6	?	1	4.4	
Wheat bran	19.1	E	4	1.3§	
Zein, commercial	91.6	E	2	1.6	

† A Acid hydrolysis.

E Enzymatic (pepsin) hydrolysis.

§ Per cent present in the protein soluble to pepsin.

Standard solutions of *dl*-methionine were prepared, and the color produced by 5 ml. aliquots was compared against distilled water in a Cenco Photometer fitted with a green filter.¹ From the data obtained a curve was plotted on semilogarithmic paper, with photometer readings as ordinates and concentration (mg./ml.) as abscissae. Because many of the hydrolyzates were of a light amber or yellow color, a modification was introduced to prevent distortion of methionine values: 5 ml. of the hydrolyzate were diluted with water to 12.3 ml., a drop of concentrated HCl was added, and this solution was used in place of distilled water as the standard against which the color was read. The diluted hydrolyzate absorbed the same amount of light (*i.e.*, gave the same photometer readings) as did 5 ml. aliquots of the same hydrolyzate which were treated with all of the color-producing reagents except the nitroprusside. These results indicated that effective compensation had been made for the color of the hydrolyzate.

Experiments in which measured amounts of methionine were added to samples before hydrolysis indicated that good recovery could be obtained, as shown in Table II. With 3 representative samples, *l*-arabinose was added as an adulterant which might interfere with the satisfactory preparation of hydrolyzates; the analytical results obtained were in good agreement with those given in Table I.

McCarthy and Sullivan (11) studied the color complex of the method in regard to its specificity as a reagent for methionine. Because of the interest in a number of compounds bearing certain relationships with methionine, tests were made in this laboratory with some other pure compounds. The concentration of the compounds tested in these studies was 0.5 mg./ml., which is equal to the maximum methionine standard used. Methionine sulfone, glutathione, choline chloride, and *d*-lanthionine² gave no color. *S*-Methylcysteine was 70% as effective as methionine in producing color (on a weight basis), while *dd*-cysta-

¹ The McCarthy-Sullivan method (11) follows: A 5 ml. aliquot was placed in a 2.5×25 cm. test tube, and 1 ml. of 14.3 *N* NaOH, 1 ml. of 1% glycine (aqueous), and 0.3 ml. of 10% sodium nitroprusside (aqueous) were added, with mixing after each addition. The tube was put in a water bath at 35–40°C. for 5–10 minutes and cooled in ice water for 2 minutes, then 5 ml. of a cold mixture of 9 volumes of concentrated HCl and 1 volume of H_3PO_4 were added, with shaking during acid addition. The tube was shaken continuously for 1 minute and cooled at room temperature for 10 minutes, after which time the color was read in the Photometer.

² The samples of *d*-lanthionine and *dd*-cystathionine were kindly provided by Dr. V. du Vigneaud.

TABLE II

Experiments Concerning the Recovery of Methionine Added to Feedstuffs and the Effects of Arabinose on the Preparation of Hydrolyzates

Method of hydrolysis	Sample	Milligrams methionine				
		Pre-viously found	Added	Total calculated	Total found	Recovery per cent
Acid	Sardine meal	10.6	10.0	20.6	20.5	99.5
	Corn gluten meal	10.5	—	10.5	10.2	97
	+ 200 mg. arabinose					
	Soybeans, raw	6.4	—	6.4	6.4	100
	+ 200 mg. arabinose					
	Meat scrap	13.6	—	13.6	12.5	92
Enzyme	+ 200 mg. arabinose					
	Meat scrap	12.6	9.6	22.2	21.3	96
	Whey, dried	9.3	9.6	18.9	17.8	94
	Alfalfa meal	8.2	9.6	17.8	18.4	104
	Corn gluten meal	10.5	9.6	20.1	19.5	97
	Sesame meal	16.3	9.6	25.9	25.0	96.5
	Soybeans, raw	7.4	9.6	17.0	17.0	100

thionine² produced 20% as high a value as methionine. Solutions of methyl thiocyanate, methyl thiourea sulfate, diethyl sulfide, and carbon disulfide turned green when acid was added in the final step; hence, comparisons with these compounds could not be made.

The results presented in Table I give an indication of the comparatively narrow range of the methionine contents of most proteins in feedstuffs. With the exception of sesame meal and sunflower seed meal, all of the common vegetable proteins analyzed contain 2.3% or less methionine; the animal protein products show more variation, but products of recognized high protein quality (*e.g.*, sardine meal) contain more methionine than those of low protein quality (*e.g.*, gelatin, fish press water).

B. BIOLOGICAL ESTIMATIONS

Biological estimations of methionine were conducted by measuring the efficiency of gain of chicks fed a diet containing raw soybeans plus additions of the feed tested. The composition of the diet was as follows: wheat bran, 10; ground wheat, 20; ground corn, 20; alfalfa meal, 4; dried brewers' yeast, 5; ground raw soybeans, 17; salt (+ trace elements), 1; ground limestone, 1.5; bonemeal, 2.0; vitamins A and D carrier, 0.25; feed to be assayed, 5-15; and corn starch to 100 parts.

S. C. White Leghorn chicks were housed in electrically heated battery brooders and reared on a stock diet for 1 to 2 weeks. They were then weighed and segregated into groups of 6-10, having a uniform average weight and weight distribution. The groups were given the experimental

TABLE III

Comparison of the Analytical and Biological Methionine Contents of Certain Feeds

Supplement assayed	Level in diet per cent	Methionine content of supplement	
		Analytical per cent	Biological per cent
Sardine meal	10	2.1	2.0
Shark meal	10	1.1	1.0
Tuna meal	10	2.0	2.0
Meat scrap	10	1.4	1.3
Sardine "stick" dried (press water)	10	1.2	1.3
Wheat protein, hydrolyzed, commercial	5	2.4	2.8
Fibrin	10	2.0	2.6
Beef blood cells, dried	10	1.3	0.9
Casein	5 }	1.8	1.9
+ Gelatin	5 }		
Alfalfa leaf meal	10	0.6	0.5
Sesame meal	10	1.6	1.7
Cottonseed meal	10	0.9	0.8
Peanut meal	10	0.7	0.7
<i>dl</i> -Methionine	0.1 }	1.4	1.2
+ Gelatin	10.0 }		
<i>dl</i> -Methionine	0.15 }	1.9	1.7
+ Gelatin	10.0 }		
<i>dl</i> -Methionine	0.15 }	2.2	2.2
+ Peanut meal	10.0 }		
<i>dl</i> -Methionine	0.20 }	2.4	1.9
+ Gelatin	10.0 }		
<i>l</i> (-)-Cystine	0.20 }	0.4	0.6
+ Gelatin	10.0 }		

diets for 8 to 12 days, during which time records of weights and feed consumption were kept.

In each series of groups, 10 parts of gelatin (methionine 0.4%) or of peanut meal (methionine 0.7%) were added to one diet which was used as a lower control; and 10 parts of casein (methionine 3.1%) or of fibrin (methionine 2.9%) were used in another diet as an upper control. Gain

per unit of feed consumed was calculated for each group and a plot made of the gain/feed against the methionine added to the diet in the form of supplement. With gelatin or peanut meal supplements the ratio of gain to feed was approximately 0.26, while with casein or fibrin the ratio usually exceeded 0.45. Methionine contents of other feeds were found by linear interpolation between the lower and upper control values in each series. Such interpolation appeared to be justified by the linear relation observed in cases where varying levels of *dl*-methionine or of other supplements were added to the diet.

The nature of the results obtained and the agreement between chemical and biological data are illustrated in Table III.

The high degree of correlation between the biological responses and the analytical methionine contents of the supplements is strong evidence that the basal diet is deficient primarily, if not exclusively, in methionine. This deficiency is created largely by the use of raw soybean protein which permits good growth when supplemented with methionine but not with cystine (3). No correlation was observed between the reported cystine contents of the feedstuffs used and the growth responses. The data of Table III are sufficiently in agreement to support the statement that the procedures used for determining methionine are substantially accurate as applied to the feeds listed.

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SUMMARY

The methionine contents of a number of feedstuffs have been determined by chemical and biological methods. The chemical determinations were made on hydrolyzates prepared by acid or pepsin hydrolysis, while the biological results were obtained by measuring the efficiency of gain of chicks fed the supplements to be tested in a diet containing raw soybeans as the principal protein source. The close correlation between the two methods indicates that the methionine determined analytically in the supplements is completely available for chick growth, and that the methods employed are substantially accurate. The cystine content of the proteins analyzed appeared to have no appreciable effect on the methionine determinations.

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The Conversion of Fructose-6-Phosphate into Glucose-6-Phosphate in Plant Extracts

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INTRODUCTION

While the intermediate reactions in the conversion of glycogen into lactic acid, in muscle extracts, and the fermentation of sugar to ethyl alcohol, by yeast preparations, have been worked out in considerable detail (see 1 for a review), the corresponding changes in higher plant tissues have been studied very little. A similar investigation of the intermediate reactions in carbohydrate metabolism in higher plants seems, therefore, desirable. The obvious approach is to study first of all the possible occurrence in higher plants of such reactions as have been reported to occur in yeast and muscle extracts. The present work is a start toward such an investigation.

Previous workers have found that phosphorylation occurs in plant extracts (3, 4, 5, 6, 7), and hexose phosphates have been isolated from various plant materials (2, 8, 9, 10). These hexose phosphates resembled those formed in yeast and muscle preparations. There have been few studies dealing with the reactions involved in the formation of these esters. Tankó (7) reported the conversion of fructose-6-phosphate into glucose-6-phosphate by suspensions of pea meal. However, few details are given, and apparently iodometric methods alone were used to detect the appearance of glucose-6-phosphate. In such suspensions of pea meal, several additional reactions probably occur. It is, therefore, conceivable that the reported changes in iodometric values may have been caused by reactions other than the conversion of fructose-6-phosphate into glucose-6-phosphate. Consequently, it seemed advisable to study this reaction in more detail. The present paper contains a more

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detailed study of this reaction. Using three independent methods of analysis we have confirmed Tankó's results and have found that sodium fluoride and sodium monoiodoacetate have no effect upon the reaction.

EXPERIMENTAL

Methods and Materials

Fructose-6-phosphate was prepared from the acid calcium salt of fructose-1,6-diphosphate which was obtained from Schwarz laboratories. The rate of hydrolysis of the latter ester in 1 *N* HCl, and aldose determinations, indicated about 5 per cent glucose-6-phosphoric acid present as an impurity. There was only a trace of inorganic P. Total P and Ca analyses indicated 76.2 and 82.9 per cent, respectively, of the theoretical values for pure anhydrous acid calcium fructose diphosphate. Drying *in vacuo* at 35°C. showed 6 per cent water in the sample. The fructose-1,6-diphosphate was hydrolyzed in 1 *N* oxalic acid according to the method of Neuberg (19). The resulting inorganic phosphate was removed with an excess of barium acetate in an alkaline solution. The filtrate was treated with mercuric acetate, and then sparingly soluble phosphoric acid esters were removed by adding enough ethyl alcohol to give a 10 per cent solution. The barium salt of fructose-6-phosphate was further purified by using essentially the procedure of Robison (11).

The purified fructose-6-phosphate resembled very closely the supposedly pure samples reported by Robison. The hydrolysis rate of a 0.01 *M* solution of the ester in 1 *N* HCl at 100°C. agreed well with the data given by Robison. The optical rotation of an aqueous solution of the ester was $[\alpha]_D^{25} = +4^\circ$ ($c = 0.5$, $l = 2$). Other analyses obtained for the ester agreed fairly well with those calculated for the barium salt with one molecule of water of crystallization: total P: *found* 7.11%, *theory* 7.51%; inorganic P: *found* 0.0%, *theory*, none; Ba: *found* 33.4%, *theory* 33.3%; H₂O: *found* 4.2%, *theory* 4.36%.

Analysis of Digests

Aliquots of the various digests were prepared for analysis by precipitating the proteins with trichloroacetic acid in a small centrifuge tube. Na₂SO₄ was added to precipitate the barium in the digest. After being thoroughly mixed, the tubes were centrifuged. Aliquots of the supernatant solutions were used for analysis. The determinations made were: inorganic phosphate, total phosphate, change in inorganic phosphate as a result of 180-minute hydrolysis in 1 *N* HCl at 100°C. ($= \Delta 180$ P), aldose, and ketose.

Total P was determined by digesting an aliquot of the appropriate solution with 2 ml. of 19 *N* H₂SO₄, using redistilled, 30 per cent hydrogen peroxide to facilitate digestion, followed by an hydrolysis of meta and/or pyrophosphoric acid that may be formed during the digestion.

The same method, omitting digestion and hydrolysis, was used to determine *inorganic phosphorus*. In this case, 5 ml. of 7.5 *N* H₂SO₄ were used instead of 2 ml. of 19 *N*. This method (21) is a modification of that given by Allen (12), and it follows the recommendation of Gomori (20) concerning the ratio of molybdate to acid. The

relative amount of molybdate is high. As a consequence, appreciable quantities of acids, such as hydrochloric and trichloroacetic, do not affect the results. The method has been used satisfactorily with esters which are as readily hydrolyzed as glucose-1-phosphate. Highly purified glucose-1-phosphate gives little or no color with the reagents over the time interval ordinarily used for analysis.

The *hydrolysis rate* of the esters used was determined according to the methods of Robison (11) and Lohmann (13).

Aldose was determined by the method of Macleod and Robison (14) using an aliquot of the trichloroacetic acid supernatant which had been carefully neutralized with NaOH to an orange color to phenol red. *Ketose* was determined by the resorcinol method of Roe (15), either using directly the trichloroacetic acid supernatants, or aliquots of the solutions neutralized for aldose determination. The results were identical in either case. In all cases, the samples were heated at 80°C. ($\pm 0.8^\circ$) for 15 minutes as recommended by Ochoa (16) for fructose-diphosphate. A standard fructose sample was run repeatedly along with experimental samples, and the results were found to be satisfactorily reproducible.

Preparation of Extracts

Seeds of the Thomas Laxton variety of peas were used. These were ground to a fine flour in the porcelain mill described by Kirk and Sumner (17). The ground meal was extracted with water. The extract was centrifuged hard and was exhaustively dialyzed against distilled water. The resulting extracts appeared cell-free under the microscope, but they contained suspended particles of undissolved protein.

TABLE I
Composition of Some Digests

Additions	Digests		
	E	I	F
1. Extract	15 ml.	15 ml.	15 ml.
2. Fructose-6-phosphate solution (5.15 mg. Ba salt/ml.)	15	15	15
3. Citrate buffer, 0.5 M, pH = 5.95	3	3	3
4. Water	1	—	—
5. 0.034 N sodium iodoacetate, pH = 6	—	1	—
6. 0.68 N, NaF, pH = 6	—	—	1
Total volume	34	34	34

Table I indicates how these extracts were used to prepare digests. Digests similar to digest E were also prepared using boiled extract. Other digests were prepared by replacing the fructose-6-phosphate solution with distilled water. The temperature was 22°C. Toluene was always added to the digests.

RESULTS

The results with duplicate digests agreed reasonably well. This was true for all the fractions for which analyses were made. A comparison

of duplicate digests is shown in Table II. These digests are similar to digest E in Table I. From the recorded data it can be seen that the values for Δ 180 P and inorganic P agree rather well. The ketose values also agree fairly well. In the case of the aldose values is observed a phenomenon which was encountered when not all of the samples were neutralized at one time. Upon standing in the cold room, the aldose

TABLE II
Comparison of Analyses in Duplicate Digests

Time	γ /mg. of digest									
	Δ 180 P		Inorganic P		Ketose (as fructose)		Aldose (as glucose)		Increase in Aldose	
<i>min.</i>	1	2	1	2	1	2	1	2	1	2
0	125	126	7	7	930	940	860	660	—	—
3	121	121	7	8	880	910	930	720	70	60
13	107	103	8	8	780	820	1000	840	140	180
30	—	85	—	11	—	680	—	940	—	280
90	54	52	16	16	520	480	1360	1130	500	470
240	44	42	26	26	450	430	1330	1190	470	530
1260	—	38	58	60	470	450	1470	—	610	—

values slowly increased until the samples were neutralized. This occurred also in aliquots from boiled extracts. Once the samples were neutralized they were stable, if protected with toluene. The samples from digest 1 were all neutralized several days later than those from digest 2. During this time the aldose values increased. Apparently some readily hydrolyzable, non-reducing, aldose-containing compound was present. This could have been sucrose that had not been removed completely by dialysis. However, this seems unlikely in view of the exhaustive dialysis. Whatever this compound was, it apparently did not change quantitatively during the experiment. At any rate, in these experiments we are primarily interested in changes in the various fractions during the course of the experiment. It will be noted that the changes in aldose are comparable in the two digests.

The data in Table II gives a general picture of the course of events in all of the digests containing fructose-6-phosphate. In all cases, Δ 180 P and ketose decreased while inorganic P and aldose increased.

This is what would have been expected if the following reactions were occurring:

1. fructose-6-phosphate \rightarrow glucose-6-phosphate.
2. hexose phosphate \rightarrow hexose + inorganic phosphate.

Since fructose-6-phosphate is hydrolyzed about ten times faster in 1 *N* HCl at 100° than glucose-6-phosphate, reaction 1 would lead to a decrease in Δ 180 P. This reaction would also cause a decrease in the ketose value as the fructose-6-phosphate disappeared and an increase in the aldose values as glucose-6-phosphate is formed.

The increase in inorganic phosphate apparently resulted from the action of phosphatase (reaction 2, above). Data for changes in inorganic phosphate are recorded in Table III. It is unlikely that any

TABLE III
*Changes in Inorganic Phosphate in the Presence and Absence of
Fluoride and Iodoacetate*

Time min.	Increase in inorganic P (γ /ml. of digest)			
	-Fluoride and -Iodoacetate	+Fluoride	+Iodoacetate	Extract only
3	1	—	-1	—
10	1	1	1	1
30	1	1	3	—
90	9	3	9	0
240	19	7	17	3
1065	49	25	45	—
1260	—	—	—	13

oxidation occurred in these extracts for it has been found in this laboratory that such dialyzed extracts of pea seeds do not reduce methylene blue.

The increase in inorganic phosphate is comparatively small in all cases during the first 90 minutes when the major changes occur in Δ 180 P (*cf.* Table II). There is some increase even when no fructose-6-phosphate is added to the extract. The increase in inorganic phosphate is lowered considerably in the presence of *M*/50 fluoride, but *M*/1000 iodoacetate has little or no effect.

The average rate of hydrolysis of the fructose-6-phosphate under the conditions of these experiments was estimated from the analytical results. It was found that 85 per cent of the ester was hydrolyzed in

180 minutes. We used Robison and Macfarlane's (18) value of 9 per cent for the degree of hydrolysis of the glucose-6-phosphate in 180 minutes. With a knowledge of these hydrolysis rates and the values we obtained for Δ 180 P in any given aliquot, it was possible to calculate the relative proportions of fructose-6-phosphate and glucose-6-phosphate. Also, the decrease in fructose-6-phosphate could be calcu-

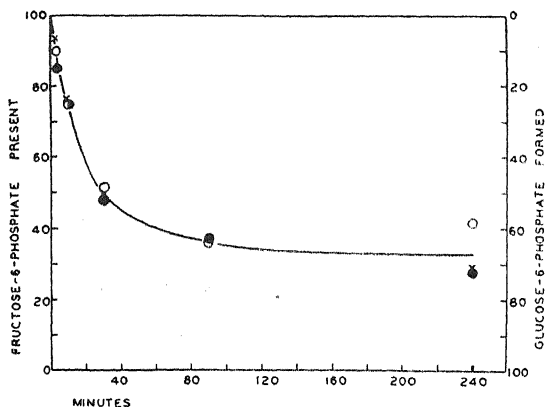


FIG. 1

Conversion of Fructose-6-phosphate to Glucose-6-phosphate

The ordinates are given in relative terms, *i.e.* as per cent of the fructose-6-phosphate added to the digest.

- × = values obtained by ketose determinations.
- = values obtained by aldose determinations.
- = values obtained by phosphorus-ester hydrolysis rate.

lated from the ketose values, and the increase in glucose-6-phosphate could be calculated from the aldose values. The results of such calculations are given in Fig. 1. It will be seen that the three independent methods of analysis give essentially the same results when calculated on this basis. After 90 minutes, the results show that 63 per cent of the added fructose-6-phosphate had been changed to glucose-6-phosphate. After 240 minutes, the corresponding value is 67 per cent. After an additional 17 hours, there was very little change. The values at that time indicated that 70 per cent of the added fructose-6-phosphate had been converted into glucose-6-phosphate. The presence of $M/50$ fluoride or $M/1000$ iodoacetate had no significant effect upon these results.

DISCUSSION

These results apparently demonstrate that fructose-6-phosphate, when added to dialyzed extracts of pea seeds, is converted into an aldose-mono-phosphate, presumably glucose-6-phosphate. It is more likely to be glucose-6-phosphate than any other aldose phosphate, in view of the results with yeast and muscle extracts and the isolation of glucose-6-phosphate from plant tissues. Under the conditions we used, the reaction proceeds until about two-thirds of the initial fructose-6-phosphate has been converted into glucose-6-phosphate, and then there is little change for long periods of time. The rather constant proportion between the two esters persists even though an appreciable amount of inorganic phosphate is formed. This inorganic phosphate apparently results from the hydrolysis of the hexosemonophosphates by phosphatases. Our results are not sufficiently refined to decide for certain which of the hexosemonophosphates is hydrolyzed, or whether or not both of them are hydrolyzed. Also, the presence of fluoride greatly decreases the phosphatase action without influencing significantly the conversion of fructose-6-phosphate into the aldose ester.

The relative proportions of ketose monophosphate and aldose monophosphate when the apparent equilibrium has been reached agree well with the relative proportions found at equilibrium in yeast and muscle extracts.

ACKNOWLEDGMENT

We wish to express our thanks, through Professor James B. Sumner, to the Rockefeller Foundation for financial support.

SUMMARY

1. Additional evidence for the occurrence of phosphohexoisomerase in plant tissues is reported. When fructose-6-phosphate is added to dialyzed aqueous extract of pea seeds, about two thirds of it disappears. Simultaneously there is formed an equivalent amount of aldose monophosphate which is considered to be glucose-6-phosphate.

2. The dialyzed pea extracts also contain a phosphatase, and, as a result, some inorganic phosphate is formed simultaneously with the conversion of the ketose ester into the aldose ester.

3. The apparent equilibrium position obtained for the conversion of fructose-6-phosphate into the aldose ester agrees with the equilibrium position reported for this reaction in yeast and muscle extracts.

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On the Inability of Intact Yeast Cells to Ferment Their Carbohydrate Reserves *

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INTRODUCTION

In the course of an investigation into the physiology of adaptive enzyme synthesis, it became necessary for the present authors to investigate the nature of the endogenous metabolism of the yeast strains employed. The data obtained supply additional information on and support for a little commented on but important observation reported by Stiller and Stannard (1935a,b; 1937), *viz.*, yeast cells which have no difficulty in fermenting external substrate, dissimilate their carbohydrate reserves by a purely aerobic mechanism.

It has been (and apparently still is) generally assumed that the metabolism of the stored polysaccharides in whole yeast proceeds *via* the same mechanisms as those postulated for the respiration and fermentation of simple carbohydrates placed in the external medium. The common term "autofermentation" has been applied to the endogenous decomposition of the carbohydrate reserves of yeast juices, dried yeast, pressed yeast, as well as to intact cells (*cf.* Harden, 1932). Our knowledge of the fermentation of externally placed sugars stems, at least in part, from comparative studies of breis and intact cells (see, *e.g.*, Hofstetter, Leichter, Nord, 1938). However, the implication that uninjured yeast cells can ferment their internally stored carbohydrate was based almost exclusively on experiments with juices and breis, or with cells injured by pressing or grinding, or with lytic agents such as toluol.

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There have existed for some time in the literature isolated observations on whole cells which appear to question the correctness of this assumption. These have taken the form of R.Q. determinations in the absence of substrate as controls for the period when substrate is added (see, e.g., Meyerhof, 1925; Warburg, 1927). Invariably the R.Q. values obtained were lower than or close to unity indicating little or no aerobic fermentation in the absence of external substrate.

The first direct and thorough investigation of the nature of the endogenous metabolism of intact yeast was made by Stier and Stannard (1936a,b; 1937). These authors found that the R.Q. was close to unity throughout a major portion of the endogenous respiration curve and tended to fall when the rate of respiration decreased to approximately $\frac{1}{4}$ of its initial value. Except for vanishingly small rates, anaerobic CO_2 production could not be demonstrated in the absence of substrate. They also found that the Q_{O_2} and $Q_{\text{CO}_2}^{\text{O}_2}$ were affected in a parallel fashion by both KCN and iodoacetic acid. On the basis of their data, they concluded that the endogenous respiration is functionally a unitary system and distinctly different from the one involved in the metabolism of external substrate. Winzler and Baumberger (1938) made a significant contribution to this problem when they showed that in the absence of substrate under anaerobic conditions suspensions of yeast cells produced little if any heat.

The investigations of Stier and Stannard (1935a,b; 1937) were carried out on two baker's yeast strains (*Saccharomyces cerevisiae*). In the present investigation a comparative study was made of six strains, two of which were *S. cerevisiae*, the other four being representatives of other yeast types. These particular strains were chosen for the present study because three of them could adapt to ferment galactose, whereas the others could not. No significant differences were found between the adaptable and non-adaptable types. It is thus not possible to refer adaptability directly to the endogenous respiration although, as will be discussed elsewhere, adaptation is not entirely independent of it.

The results obtained indicate that the conclusions arrived at by Stier and Stannard from their studies on the two bakers' yeast strains are valid for other yeast genera and species as well. Certain differences were found particularly in the time course of the endogenous metabolism. These will be discussed. However, the endogenous metabolism of all six yeasts was found to be purely respiratory in nature and none of them could ferment their carbohydrate reserves to any appreciable extent.

MATERIALS AND METHODS

(a) *Yeast Strains*. Two strains of *Saccharomyces cerevisiae* (LK2G12 and 812) were used in the present study. Both of these strains can adapt to galactose fermentation by direct interaction with the substrate. (For origin and characteristics of these two strains see Spiegelman, Lindegren, Hedgecock, 1944; Spiegelman, Lindegren, 1944a.) The other adaptable strain used was *Saccharomyces carlsbergensis* (Cl.). The three unadaptable strains employed were *Schizosaccharomyces pombe* (P1), *Schizosaccharomyces octosporus* (O1), *Saccharomycodes ludwigii*¹ (L1). The non-adaptive characteristics of the last three strains are discussed in another paper (Spiegelman, Lindegren, 1944b). All of the above strains are diploid, as evidenced by their colonial stability and their ready sporulation.

(b) *Media*. The culture medium was made by adding the following to 1 liter of water: 2 g. of autolyzed yeast extract powder, 5 g. of Bacto peptone, 1 g. $(\text{NH}_4)_2\text{SO}_4$, 2 g. of KH_2PO_4 , 0.25 g. of MgSO_4 , 0.13 g. of CaCl_2 , 7 cc. of 50 per cent sodium lactate, 80 g. of dextrose. After being brought to a boil, the mixture is cleared by filtration and dispensed into 250 cc. capacity Erlenmeyer flasks in 75 cc. quantities for sterilization and use.

(c) *Methods of Handling Cultures*. One of the problems always encountered in making physiological studies of yeast strains is the danger of introducing genetic variants. With diploid cultures this is reduced to a minimum, providing sporulation can be suppressed. It was found that the maintenance of high carbohydrate concentrations and frequent transfers reduce sporulation to undetectable levels. Stock cultures of the strains used in the present study were carried in the 8 per cent dextrose medium described above and were transferred every 48 hours. Microscopic examinations were made periodically for evidence of sporulation. Under these conditions none was found. All cultures were incubated at 29°C.

(d) *Manometric Measurements*. The oxygen consumption, and aerobic and anaerobic CO_2 production were measured in Warburg vessels shaken at a rate of 110 complete oscillations per minute through a 7 cm. arc. All measurements were done at 30.2°C. The aerobic CO_2 production was obtained by the usual two-cup method. The anaerobic CO_2 production was determined by including a third vessel without KOH, in which the air was replaced by nitrogen from which the last traces of oxygen were removed by passage over hot copper. Inaccuracies due to retention of CO_2 were reduced to a minimum by using $M/15 \text{ KH}_2\text{PO}_4$ as the suspending medium in all cups.

(e) *Standard Suspensions*. In determining the whole course of the endogenous respiration, it is of some importance to start measurements as soon as possible after removal of the cells from the medium. To facilitate the rapid preparation of suspensions of the desired density, a photoelectric colorimeter was calibrated so that readings could be interpreted in terms of mg. of dry weight per cc. of suspension. Since the optical properties of these suspensions differed, a separate calibration curve was necessary for each strain used, and each such curve was based on a 48-hour culture. On every third run, samples were taken for dry weight as a check against the calibration

¹ The last four yeast strains mentioned were obtained from the collection of the Northern Regional Research Laboratory at Peoria, Illinois, through the courtesy of Dr. L. J. Wickerham.

curve. The density used was such that each cup contained between 4-8 mg. dry weight of yeast. This was however, increased in experiments in which respiratory inhibitors were used.

The yeast was centrifuged in 50 cc. tubes at approximately 2500 r.p.m. for 7 minutes. After allowing the medium to drain out, $M/15$ KH_2PO_4 was carefully poured down the side so as not to disturb the yeast, and was then poured out and allowed to drain. This served to wash the medium from the sides of the tubes. The cells were then resuspended and centrifuged. This procedure was repeated once more. After this, the washed cells were resuspended in $M/15$ phosphate and adjusted to the desired density. Between 30 and 40 minutes elapsed from the time of removal from the medium and the taking of the first reading.

(f) *Dry Weights.* 5 or 10 cc. portions of the experimental suspensions were pipetted into absolute alcohol contained in Jena 164 glass filters. The yeast cells were separated from the alcohol by suction and washed with several volumes of 30 per cent alcohol. The weighing vessels were allowed to dry in an air oven at $70^\circ C$. and then removed to a desiccator and allowed to stand until they came to constant weight.

(g) *Physiological State of the Cells.* At the end of every manometric experiment, microscopic examinations were made of samples from the suspension in the cups. The methylene blue technique was utilized to determine the number of dead cells. The number of cells taking up the dye was always less than 1 in 500, in all the experiments reported. No bacterial growth was noted in any of the experiments as tested by either direct examination of stained smears or streak plates made from samples of the suspensions.

EXPERIMENTAL RESULTS

A. *Metabolic Characteristics of the Yeast Strains.* A comparison was made of the metabolic characteristics with and without external substrate. The results are summarized in Table I. The Q_{O_2} and $Q_{CO_2}^O$ and $Q_{CO_2}^N$ were all determined on 48-hour cultures. The values obtained

TABLE I

Metabolic Characteristics of Various Yeasts

All measurements were made on 48 hour culture at $30.2^\circ C$. The values in the absence of substrate were obtained in the constant rate portion of the endogenous respiration curve.

Culture	Number of expts.	Without substrate				With substrate (4% glucose)			
		Q_{O_2}	$Q_{CO_2}^O$	R.Q.	$Q_{CO_2}^N$	Q_{O_2}	$Q_{CO_2}^O$	R.Q.	$Q_{CO_2}^N$
LK2G12	32	30.0 ± 0.5	30.1 ± 0.4	1.00	0.2 ± 0.05	54.2 ± 2.2	203 ± 10.5	3.75	344 ± 9.4
S12	32	28.1 ± 1.1	28.0 ± 1.5	1.00	0.1 ± 0.1	61.0 ± 3.5	194 ± 9.7	3.18	285 ± 6.3
C1	20	31.0 ± 1.8	31.0 ± 2.1	1.00	0.4 ± 0.1	82.3 ± 4.9	208 ± 6.1	2.54	281 ± 5.9
O1	15	21.0 ± 0.9	21.0 ± 1.4	1.00	0.1 ± 0.06	90.2 ± 1.8	159 ± 6.4	1.76	225 ± 8.5
P1	12	17.9 ± 1.2	17.7 ± 1.9	0.99	0.4 ± 0.1	36.4 ± 1.8	169 ± 5.1	4.64	220 ± 4.6
L1	10	38.0 ± 2.3	38.2 ± 2.8	1.01	0.9 ± 0.2	144 ± 6.4	360 ± 10.8	2.50	375 ± 12.8

without substrate were in all cases measured within the first hour, before the respiratory rate began to fall.

It will be noted that all the strains can ferment external substrate both aerobically, as indicated by the high R.Q. values, and anaerobically. On removal of external substrate, the R.Q. drops to unity and the rate of anaerobic CO_2 production falls to vanishingly small values in all cases. It is evident from these data that the relatively active endogenous metabolism is strikingly different from that employed in the utilization of external substrate. There can be no doubt on the basis of the R.Q. and $Q_{\text{CO}_2}^{\text{N}}$ values in the presence of glucose, that all the strains examined possess an active fermentative mechanism.

B. *The Time-Course of the Endogenous Metabolism.* A study of the changing behavior of the endogenous metabolism with time was made in dissimilating suspensions of all six strains. These experiments were performed in two different ways. Suspensions of washed cells were made in $M/15 \text{ KH}_2\text{PO}_4$ and shaken at 110 oscillations per minute through a 7 cm. arc in a constant temperature bath at 30.2°C . Samples were taken out at intervals for measurements of Q_{O_2} and $Q_{\text{CO}_2}^{\text{O}}$ and $Q_{\text{CO}_2}^{\text{N}}$. The other method consisted in following the rate continuously in the Warburg vessels. No significant difference was found between the results obtained by the two methods.

The results obtained with the six cultures are given in Figs. 1-6. All the cultures start out with a relatively high respiratory rate, which is maintained for from 60 to 120 minutes. After this constant rate period, a rapid decline occurs, at the end of which the Q_{O_2} and $Q_{\text{CO}_2}^{\text{O}}$ approach zero asymptotically. The R.Q. during the constant rate period is either unity or close to it. However, it will be noted that in all cases there is a tendency for the R.Q. to fall to lower values after a considerable decrease in the rate of oxygen consumption has occurred. This fall in R.Q., with low Q_{O_2} , is apparently quite common (*cf.* Meyerhof, 1925; Stier and Stannard, 1935a; Cook and Morgan, 1940). The $Q_{\text{CO}_2}^{\text{N}}$ values were followed throughout the periods represented in Figs. 1-6, but were not included since they at no time exceeded the values already recorded for the various strains in Table I.

In the present study, and in others on the relation between adaptive enzyme and the endogenous respiration to be published subsequently, use is made of the constant rate portion of the endogenous curve. It was thus of some interest to determine the relation between it and the declining portion of the curve. It has been generally assumed (Harden

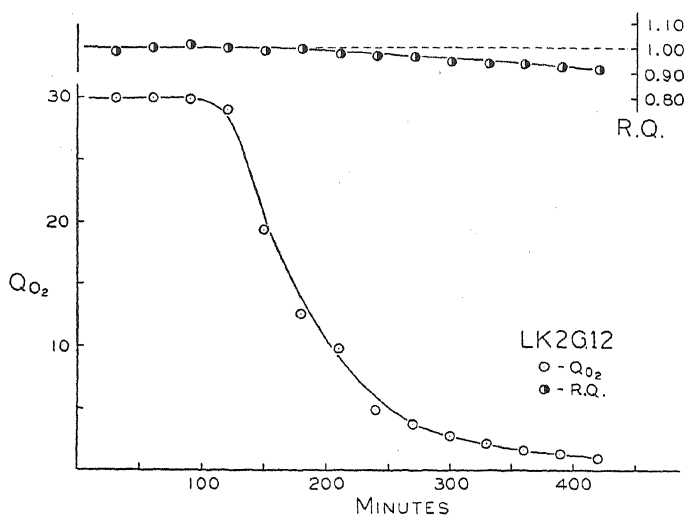


FIG. 1

Respiration and R.Q. during Dissimilation of a 48 Hour Culture of a *Saccharomyces cerevisiae* Strain

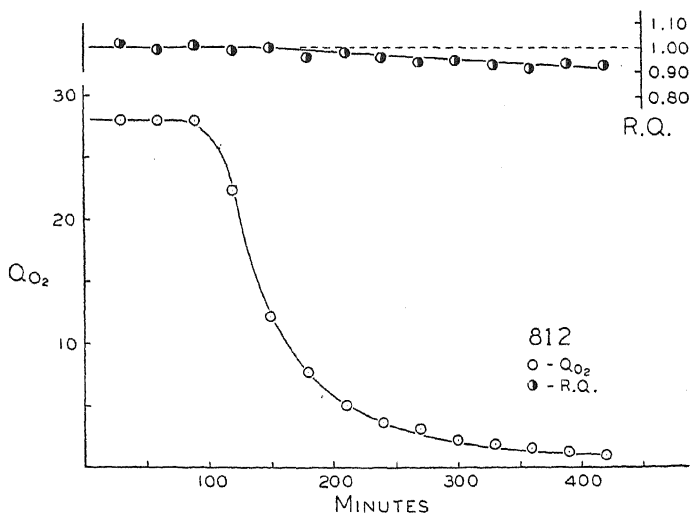


FIG. 2

Respiration and R.Q. during Dissimilation of a 48 Hour Culture of a *Saccharomyces cerevisiae* Strain

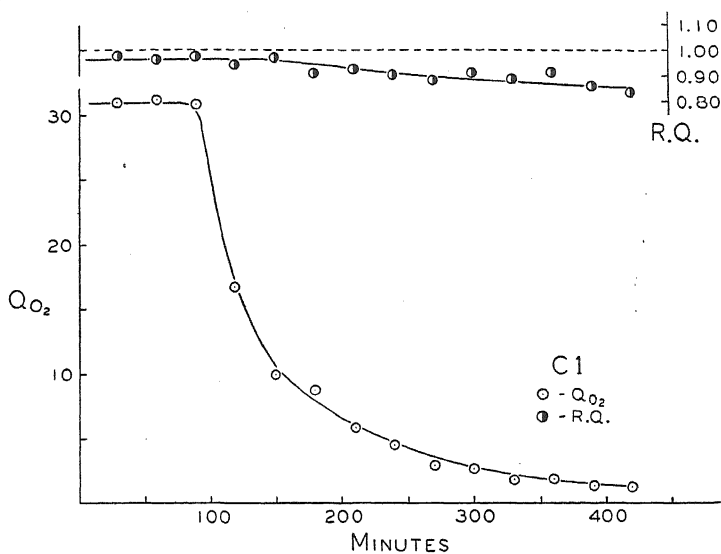


FIG. 3

Respiration and R.Q. during Dissimilation of a 48 Hour Culture of a *Saccharomyces carlsbergensis* Strain

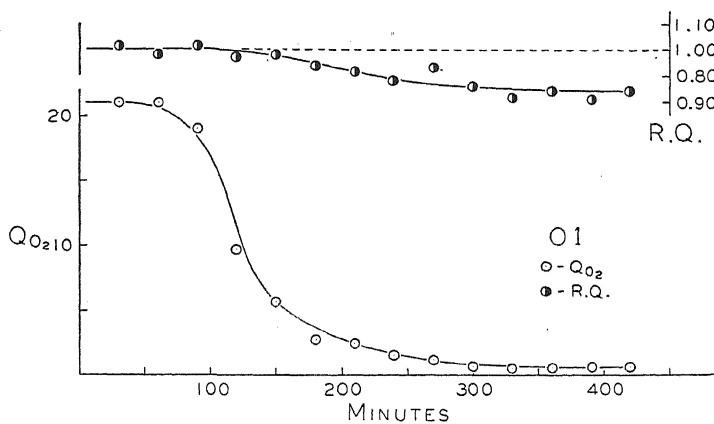


FIG. 4

Respiration and R.Q. during Dissimilation of a 48 Hour Culture of a *Schizosaccharomyces octosporus* Strain

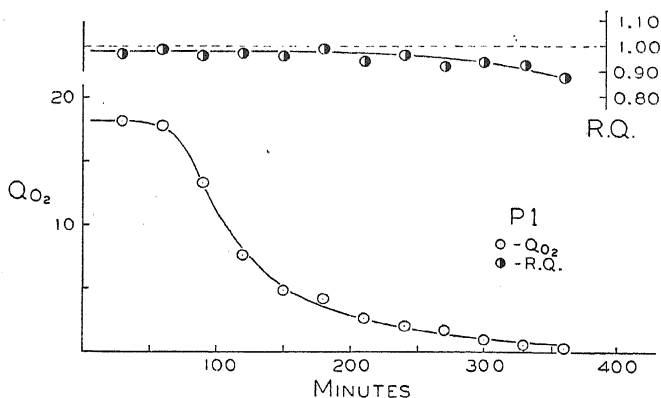


FIG. 5

Respiration and R.Q. during Dissimilation of a 48 Hour Culture of a *Schizosaccharomyces pombe* Strain

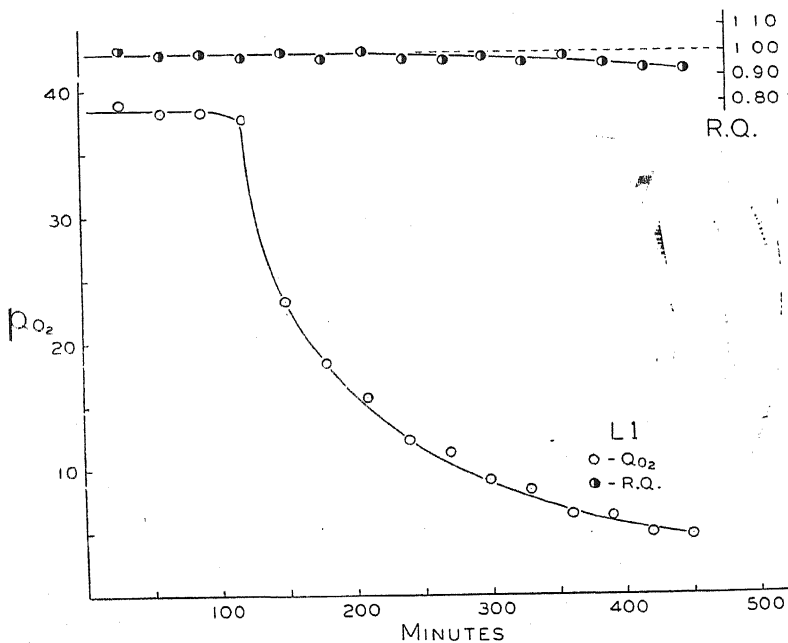
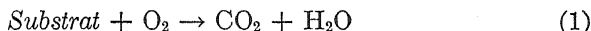


FIG. 6

Respiration and R.Q. during Dissimilation of a 48 Hour Culture of a *Saccharomyces ludwigii* Strain

and Paine, 1912; Harden, 1932; Belitzer, 1934) that the fall in respiratory rate with time is due to substrate exhaustion rather than to a gradual deterioration of the enzymes involved. This suggestion is supported by the data of Stier and Stannard (1935a), who assume a first order decomposition of the substrate. The data obtained in the present study can generally confirm this view of the nature of the decline.

On the basis of the unity of the R.Q., it seems reasonable to suppose with Stier and Stannard (1935a) that the stored substrate, *Substrat*, decomposes into CO_2 and H_2O , and the following may then be written as the basic reaction of the endogenous respiration,



Letting x equal the O_2 consumed or CO_2 produced at any time, and assuming according to (1) a proportionality between the amount of substrate metabolized and oxygen consumed, we may write

$$\frac{dx}{dt} = k(A - x) \quad (2)$$

where A is the total amount (asymptotic value of the oxygen consumed) and k is the velocity constant. Assuming no oxygen consumption at zero time and integrating yields

$$x = A(1 - e^{-kt}) \quad (3)$$

Upon taking logarithms and rearranging, one obtains

$$\ln \left(1 - \frac{x}{A} \right) = -kt \quad (4)$$

which is the equation used by Stier and Stannard (1935a). Plotting $\ln \left(1 - \frac{x}{A} \right)$ against t should then lead to a straight line with a negative slope if the declining respiratory rates are due to gradual substrate concentration limitations. Calculations based on data obtained with LK2G12 are given in Table II and the required plot is exhibited in Fig. 7. It is seen that the desired linearity for the declining portion of the curve (beyond 90 minutes) is present. A break occurs at 90 minutes which corresponds to the end of the constant rate phase. This break has always been observed in cultures which give linear plots for the

TABLE II
*Data and Calculations Testing First Order Nature of the Decline in Respiratory
 Rate of a Dissimilating 48 Hour Culture of LK2G13*

Time-min.	X-cmm.	X/A	1 - X/A	$\log \left[\left(1 - \frac{X}{A} \right) \times 10^2 \right]$
30	75	0.156	0.844	1.926
60	150	0.312	0.688	1.838
90	220	0.489	0.511	1.708
120	290	0.645	0.355	1.550
150	340	0.755	0.245	1.389
180	375	0.833	0.167	1.223
210	390	0.869	0.131	1.117
240	441	0.920	0.080	0.900
270	455	0.944	0.056	0.750
300	460	0.960	0.040	0.600
330	465	0.974	0.026	0.410

The asymptotic value, A, was equal to 480 mm³.

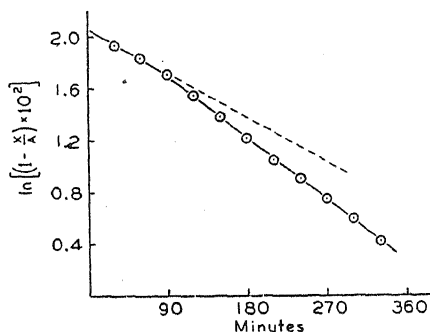


FIG. 7

Test for First Order Nature of the Declining Rate Portion of the
 Endogenous Respiration

See text for further details.

declining portion and is due to the fact that our cultures always exhibit a constant rate phase.

It must be noted that the linearity obtained is not invariable, failing with older cultures and with certain haploid strains observed in this laboratory. It is likely that in certain cases the shape of the rate-time curve is complicated by the loss of diffusible elements.

With the exception of strain P1, the initial Q_{O_2} and $Q_{CO_2}^0$ values recorded here are considerably higher than those reported in the liter-

ature which latter range from about 6 to 15. A further difference exists in the relatively long constant rate period at this high level of activity. These two differences are probably not unconnected and may be ascribed either to strain differences, methods of cultivation (8 per cent rather than the usual 2 per cent carbohydrate was used), or perhaps to both. However, Stier and Stannard (1935a) contend that the normal course of events for young cultures should follow an immediate decline with time from the very first point. In this, they are supported by Geiger-Huber (1934), who reports on initial rapid decline in the respiratory rate. Winzler and Baumberger's (1938) data on the rate of heat production as a function of time by dissimilating yeast cultures also show an immediate drop in rate. Our efforts to reproduce these immediate drops in rate with our strains, by using 16-hour cultures in 2 per cent carbohydrate, led to such variable results that we found them useless for quantitative study.

There are several reasons why it is believed that the high constant initial rate observed here is, like the remainder of the curve, endogenous in nature and is not referable to the utilization of incompletely removed external substrate. In the first place, the amount of oxygen consumed and CO_2 evolved during this period is considerable, ranging from 130 to 400 mm^3 . This would be equivalent to the complete oxidation of between 0.2 and 0.6 mg. of glucose which is certainly more than a trace. Further, in the preliminary experiments investigating the procedures employed it was found that occasionally with one washing, abnormally high Q_{O_2} values were obtained which were attributable to incomplete removal of the medium. But these were always accompanied by high R.Q. values as well as anaerobic CO_2 production, indicating exogenous metabolism. With two washings these were never observed, and three washings, except for the additional 10 minutes required, did not change the initial Q_{O_2} nor the duration of its maintenance. Finally, if this constant rate portion of the endogenous respiration is the result of the consumption of "residual hexose," it is markedly different in character from the metabolism of the added hexose and is indistinguishable from the rest of the endogenous curve except in the level of its rate. The R.Q. is unity, and the anaerobic CO_2 production during this period as elsewhere is vanishingly small.

In view of the fact that, except for its constancy and higher rate, this early portion of the endogenous respiration resembles the remainder of the endogenous metabolism in all other respects, a more reasonable

interpretation of it is available. Its existence may be interpreted as being due to the fact that the concentration level of the reserves is initially sufficiently high as to be non-limiting. The entire rate-time curve may then be expressed in terms of a typical substrate concentration-rate relation with a fixed amount of enzyme.

C. *The Effect of Cyanide on the Endogenous Respiration.* It was desirable to test further the non-fermentative nature of the endogenous respiration indicated by the R.Q. and the low $Q_{CO_2}^N$ values. Experiments were performed which compared the effects of various concentrations of KCN on the Q_{O_2} and $Q_{CO_2}^O$ values. For the purposes of easier comparison, all experiments were done in the constant rate period.

The procedure in these experiments differed from the one usually employed. The suspending fluid for both controls and experimentals was *M/15* phosphate buffer adjusted to pH 6.5. The cup which measured the oxygen consumption contained appropriate mixtures of KCN and KOH in the center well following Krebs (1935). The amount of yeast was raised to from 12 to 20 mg. dry weight per cup at the high cyanide concentrations. The data obtained with all strains are summarized in Table III.

It is clear from the table that in all the strains tested the reaction of the endogenous metabolism is typical of a purely aerobic process. No part of the aerobically produced CO_2 is apparently fermentative since the Q_{O_2} and $Q_{CO_2}^O$ were equally affected.

D. *The Effect of Iodoacetic Acid (IAA) on the Endogenous Metabolism.* Lundsgaard (1930) demonstrated the specific effect of low concentrations of IAA on the fermentative process of yeast. He found that 10^{-4} molar IAA completely inhibits the fermentation of dried yeast, leaving the respiration unhampered, the R.Q. approaching unity. This offered another method of testing for the existence of fermentative components in the endogenous metabolism.

Experiments were performed to see whether the aerobic CO_2 output was more sensitive to IAA than the oxygen uptake. Again, to avoid comparisons of rates during the period when the Q_{O_2} is decreasing with time, the observations were confined to the constant rate interval of the rate-time curve. In this case, there was a difficulty introduced by the penetration period of IAA. After some preliminary experiments it was decided that 50 minutes was sufficiently long for the IAA to exert its effect. This was at the same time short enough to be well

TABLE III

Effect of Cyanide on Constant Rate Portion of Endogenous Respiration

Culture	KCN		Inhibition		
	Molar concentration		per cent		
		Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$
812	0	27.3	27.0	—	—
	2×10^{-4}	15.4	16.0	43	41
	2×10^{-3}	0.7	1.9	97	93
	2×10^{-2}	0.0	0.0	100	100
LK2G12	0	31.2	30.0	—	—
	2×10^{-4}	20.0	19.8	38	36
	2×10^{-3}	3.4	5.8	89	85
	2×10^{-2}	0.0	0.3	100	99
C1	0	32.4	33.0	—	—
	2×10^{-4}	14.3	13.3	56	59
	2×10^{-3}	5.5	6.6	83	80
	2×10^{-2}	0.6	1.3	98	96
P1	0	17.8	18.1	—	—
	2×10^{-4}	12.7	13.6	29	25
	2×10^{-3}	6.6	6.3	63	65
	2×10^{-2}	1.8	2.2	90	88
L1	0	38.7	40.1	—	—
	2×10^{-4}	14.0	13.6	64	67
	2×10^{-3}	3.1	4.5	92	89
	2×10^{-2}	1.2	2.9	97	93
O1	0	21.4	21.0	—	—
	2×10^{-4}	14.4	14.0	33	33
	2×10^{-3}	5.6	6.1	74	71
	2×10^{-2}	0.4	1.0	98	95

within the constant-rate portion of all the endogenous curves. Table IV summarizes the data obtained in these experiments. All the values are those attained in the 20 minutes following the 50 minute wait after the IAA was added from the side arm. Here again it is seen that in all the strains the Q_{O_2} and $Q_{CO_2}^{O_2}$ parallel each other and no specific inhibitory effect on CO_2 output is observable. It is worthy of mention here that in none of the experiments, where both rates were affected, was there any time difference between the action of the IAA on the Q_{O_2} and $Q_{CO_2}^{O_2}$. Such time differences are often observed when yeast cells are metabolizing external substrate, the $Q_{CO_2}^{O_2}$ being the first to decrease.

TABLE IV

*Effect of IAA on Constant Rate Portion of Endogenous Respiration*The values reported represent those attained 50 minutes
after the introduction of the IAA.

Culture	IAA		Inhibition		
	Molar concentration		per cent		
		Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$
812	0	28.1	28.1	—	—
	2×10^{-4}	28.2	28.1	0	0
	2×10^{-3}	9.0	8.4	68	70
	2×10^{-2}	0.0	0.0	100	100
LK2G12	0	31.0	31.0	—	—
	2×10^{-4}	31.1	30.8	0	0.6
	2×10^{-3}	8.4	7.7	73	75
	2×10^{-2}	0.0	0.0	100	100
C1	0	31.8	31.0	—	—
	2×10^{-4}	31.0	31.0	1.2	0
	2×10^{-3}	11.8	9.8	63	68
	2×10^{-2}	0.0	0.0	100	100
P1	0	17.0	17.0	—	—
	2×10^{-4}	17.0	17.0	0	0
	2×10^{-3}	3.7	3.9	78	76
	2×10^{-2}	0.0	0.0	100	100
L1	0	40.1	39.2	—	—
	2×10^{-4}	40.0	38.0	2.4	3.1
	2×10^{-3}	7.0	5.5	83	86
	2×10^{-2}	0.0	0.0	100	100
O1	0	20.5	21.2	—	—
	2×10^{-4}	20.5	21.2	0	0
	2×10^{-3}	6.4	5.9	69	71
	2×10^{-2}	0.0	0.0	100	100

These experiments thus confirm the conclusions drawn from the results with cyanide that the endogenous metabolism is a purely aerobic process.

F. *The Effect of Periods of Anaerobiosis on the Rate of Endogenous Metabolism.* On the basis of the evidence presented thus far, it may be supposed that the withdrawal of oxygen would lead to a cessation of the utilization of the endogenous reserves. This was tested in all the strains by examining the effect of periods of anaerobiosis on the subsequent respiration.

Suspensions were made of 48-hour cultures which were divided into two portions. One portion was allowed to dissimilate in the usual way while shaking in the 30.2°C. bath, and samples were removed at intervals for measurement of respiratory rates. The other portion was divided into four equal parts which were placed in Erlenmeyer flasks, provided with two-hole rubber stoppers, which permitted flushing with nitrogen. These four flasks were then shaken in the 30.2°C. bath. At intervals, one after the other was opened and samples removed for measurements of respiratory rates. Once such a flask was opened it was discarded.

The results of these experiments are given in Table V. It will be noted that, as was found previously (see Figs. 1-6), within five hours under aerobic conditions both Q_{O_2} and $Q_{CO_2}^O$ fell to about 1/10 of their original values. The decrease continued so that within 25 hours the majority of them respired at 1/100 or less of their initial rate. However, the same cultures held under anaerobic conditions for the same periods of time showed no decrease. Thus, even those cultures which were held for 25 hours anaerobically, possessed after that period rates equal to those of the initial period though in the comparable aerobic cultures the rates had by that time decreased almost to zero. These experiments again offer support to the conclusion that the endogenous metabolism is purely aerobic, since there was no apparent utilization of the stored reserves during the relatively long anaerobic periods.

Although these experiments were not designed to investigate it, it is of some interest to point out a phenomenon which is apparent in Table V. It will be noted that in many cases the rates measured following the anaerobic period were higher than the one recorded initially. In some instances they are in excess by almost 40 per cent. That this phenomenon is real has been shown by other experiments whose results will be discussed elsewhere. This burst of activity is temporary, rarely exceeding 50 minutes, following which the respiratory rate returns to the original endogenous value. It is interesting to note that Winzler and Baumberger (1938) reported a burst of heat production following the introduction of oxygen to yeast suspension kept under nitrogen. As in the case of the respiratory rates, the rate of heat output, after variable lengths of time, returned to the endogenous level. The same phenomenon has been observed by Hofstetter, Leichter, Nord (1938) with both extracts and whole yeast cells.

It does not seem likely, on the basis of the evidence presented here,

TABLE V
The Effect of Periods of Anaerobiosis on the Rate of Endogenous Respiration

All values are based on the reading obtained in the first 30 minutes after equilibration.

Cultures	Hours under aerobic conditions										Hours under anaerobic conditions									
	0		5		10		15		25		0		5		10		15		25	
	Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$
LK2G12	30.0	30.2	2.4	1.9	1.8	1.4	0.9	0.7	0.1	0.1	30.0	30.2	30.8	30.5	34.5	34.0	31.0	30.6	32.6	33.0
812	28.0	28.1	2.2	2.0	0.8	0.5	0.6	0.5	0.6	0.5	28.0	28.1	26.5	27.0	30.4	30.4	36.9	35.7	31.9	32.2
CI	31.3	30.9	3.2	2.8	2.2	1.7	0.1	0.1	0.2	0.1	31.3	30.9	39.6	40.2	32.5	32.4	31.0	31.2	31.0	31.0
PI	18.2	18.2	1.3	1.0	0.4	0.3	0.0	0.0	0.0	0.0	18.2	18.2	18.7	18.2	24.6	24.3	22.0	22.9	22.6	22.0
LI	40.2	38.6	10.2	9.3	3.8	3.1	0.5	0.4	0.5	0.4	40.2	38.6	38.6	38.0	43.4	44.2	42.4	42.0	41.8	40.7
OI	22.4	23.1	0.4	0.3	0.6	0.5	0.3	0.2	0.0	0.0	22.4	23.1	30.4	31.2	24.6	23.8	24.7	24.7	23.0	23.6

that this initial burst in activity could be due to the accumulation of hexose phosphate through the phosphorylation of glycogen. Hexose phosphate is a fermentable substrate, and no rise in $Q_{CO_2}^N$ above its very small rate has ever been observed in yeast suspensions, even after long periods of continuous aerobiosis. Furthermore, it will be noted that the R.Q. of the higher respiratory rate following anaerobiosis is still close to unity and there is no evidence that a fermentable substrate is accumulating.

DISCUSSION

All the data on the six strains examined are consistent with the hypothesis that the endogenous metabolism in yeast is a purely aerobic oxidation. The existence of this purely aerobic metabolism further strengthens the already impressive collection of evidence against the view that carbohydrate utilization begins only after its anaerobic breakdown to pyruvate. In this connection, attention should be called to the work of Nord (1940) and his collaborators on carbohydrate fermentation in *Fusaria*. They found a non-phosphorylating mechanism in the initial period of contact with the carbohydrate, as well as evidence for the direct fermentation of such disaccharides as maltose (Nord and Engel, 1938) and trehalose (O'Connor, 1940).

It seems necessary to postulate that the endogenous utilization of the stored carbohydrate passes through an entirely different mechanism than that employed in the metabolism of externally placed glucose. It is probable that a similar oxidation, without previous fermentation, is involved in some other cases reported by purely aerobic carbohydrate utilization. Among these may be cited Loebel's (1925) study of the oxidation of fructose by brain tissue, Barker, Shorr, Malann's (1939) investigation of the continued respiration in the presence of IAA, and similar findings in yeast by Lundsgaard (1930). In bacteria, Barron and Friedemann (1941) present evidence in the form of some bacterial species which cannot ferment glucose but which have no difficulty oxidizing them. Van Niel and Cohen (1942) reported a similar phenomenon in the case of *Candida albicans* metabolizing glucose and sucrose.

Dickens (1936, 1938) and Lipmann (1936) have proposed a scheme of carbohydrate oxidation in which the first stages are the formation of hexose-6-phosphate and the oxidation of this to 6-phosphohexonate, then to 2-keto-phosphohexonate, and the decarboxylation of the latter

to a pentose phosphate. Warburg and Christian (1933, 1936, 1939) isolated the enzyme components involved in the oxidation of the hexose phosphate, and these were found to be protein, triphosphopyridine nucleotide, and alloxazine. This enzyme system would not be cyanide-sensitive and hence could not be the direct mediator between substrate and oxygen in the case of the endogenous respiration. To explain the cyanide sensitivity of the endogenous respiration it would be necessary to postulate a linkage between it and the cytochrome system. A link of this kind may possibly be provided by the recent report by Haas, Horecker, Hogness (1939) of the presence in yeast of a flavin-mono-nucleotide protein which acts as an electron mediator between triphosphopyridine nucleotide and cytochrome C.

Whether or not the Lipmann-Dickens scheme outlined above is applicable to the endogenous metabolism, it is impossible to say without further experiments. Whatever the details of this aerobic mechanism turn out to be, it is functionally disconnected, insofar as the endogenous substrate is concerned, from the fermentative apparatus also possessed by these cells. Apparently there is no "Pasteur Shunt" for the internally stored carbohydrate. The fact that destruction of the structural integrity of the cell leads to the fermentation of the endogenous reserves would imply that the functional disconnection between the aerobic and fermentative mechanisms is accomplished through a geometrical orientation of the substrate. This orientation apparently shunts the internal substrate molecules into the purely aerobic channels and keeps them away from the fermentative system. This view is further supported by the fact that substrate coming from the exterior and upon which less severe geometrical constraints are placed can get to the fermentative system.

On the basis of the continued aerobic respiration in cells whose fermentative system has been poisoned with IAA, it seems likely that the external substrate molecules can also be metabolized *via* the aerobic mechanism. Further evidence on this point, which will be discussed in a later paper, is the ability of yeast cells to oxidize galactose which are not adapted to its fermentation. The data indicate that this pure oxidation of galactose in the pre-adaptive period proceeds through the same oxidative system as that employed in the endogenous respiration.

One other problem of basic biological importance brought up by these experiments may be mentioned here. In the absence of external substrate, under anaerobic condition, the yeast cell produces no meas-

urable heat and very little CO_2 . Despite this almost complete lack of metabolism, it can, under such conditions, maintain its physiological integrity for very long periods of time (168 hours, which is approximately equivalent to the time required for 112 generations). In point of fact, as will be shown and discussed elsewhere, certain unstable enzymes remain fixed under anaerobic conditions. These facts are difficult to understand from the point of view of the concept that a continual flow of matter and energy is necessary for maintenance of the status quo in a living system.

SUMMARY

The endogenous respiration of six strains of yeast was examined. Two of the six were representative of *Saccharomyces cerevisiae*. The other four were *Saccharomyces carlsbergensis*, *Schizosaccharomyces pombe*, *Schizosaccharomyces octosporus* and *Saccharomyces ludwigii*. It was found that in all cases the dissimilation of the carbohydrate reserves occurred through a purely aerobic mechanism. None showed any ability to ferment their internally stored carbohydrate, although all possessed active fermentative systems for glucose. The implication of these results for the problem of carbohydrate metabolism and the influence of structural integrity on cell physiology are discussed.

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Digestibility by Rats of α -, β -, and Neo- β -Carotenes in Vegetables

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INTRODUCTION

Experiments to determine the digestibility of carotenes are limited in number and the results are conflicting. Yellow pigments other than carotene have interfered with the determination of carotene in excrements (2). Wilson, Das Gupta, and Ahmad (10) reported that humans digested 80 to 90% of carotene in raw carrots and cooked spinach when the diet contained a normal amount of fat and about 50% from spinach when fat was given. Kemmerer and Fraps (2) found that carotene in oil was digested by rats to a greater extent than carotene in alfalfa. Van Eekelen and Pannevis (8) claimed 1 to 5% carotene in carrots and spinach, and 41% of the carotene dissolved in oil was digested by humans. Virtanen and Kreula (7) also reported that only 2 to 5% of the carotene was absorbed from finely grated carrots, and the percentage of carotene and vitamin A absorbed from butter was high. Ramasarma (5) stated the digestibility by rats of carotene dissolved in oil to range from 64 to 85%. Wald, *et al.* (9) reported that carotene fed in cottonseed oil to human subjects was excreted to the extent of 60%.

Carotene, as commonly determined by various methods, has been found by Kemmerer and Fraps (3, 4) to contain β -carotene, neo- β -carotene B and U, as well as impurity A and sometimes α -carotene. Fraps and Meinke (1) have reported that rats about 21 to 40 days old digested β -carotene, neo- β -carotene B, and α -carotene to the amount of 50% when 60 μ g. of a natural mixture of these carotenes was fed daily.

The following work reports digestion experiments with adult rats. The term *digestibility* is used here to mean the difference between the

amounts of carotene fed and the amounts excreted. Some of the carotene not excreted may, however, be destroyed rather than being digested and assimilated.

EXPERIMENTAL

Male adult white rats, between 14 and 22 months of age and weighing 300 and 400 g., were used as experimental animals. For each test, 4-6 rats were placed in individual cages, given distilled water and fed a basal ration very low in vitamin A. This consisted of white corn meal 37%, wheat gray shorts 25%, calcium carbonate 1%, cottonseed meal 20%, casein 12%, yeast (irradiated) 3%, tricalcium phosphate 1%, and salt 1%. This ration contained approximately 3.8% fat, which is comparatively low. The rats received daily, in addition, a weight of food containing approximately 20 μ g. crude carotene in one series of experiments and 60 μ g. crude carotene in another series. After a preliminary period of 3 days, the feces were collected daily for 4 days, combined for each test and stored in the refrigerator. Crude carotene was determined (3) and the carotenoid constituents of the crude carotene ascertained by chromatographic absorption (3) on calcium hydroxide. Each analysis is the average of two or more repetitions.

RESULTS

The average constituents of the carotenes in the foods used in the tests are given in Table I, and the average digestibility of the carotenes having vitamin A potency in Table II. The average digestibility of β -carotene by young rats just after weaning, as previously reported (1), was 52%, and that of α -carotene was 53%, being about 50% greater

TABLE I
Constituents of the Crude Carotene (per cent)

Number averaged	Name	Crude Carotene p.p.m.	Impurity A	Neo- β -Carotene U	β -Carotene	Neo- β -Carotene B	α -Carotene	β -Carotene equivalent
2	Apricots	16	29	0	66	5	0	69
1	Beet greens	81	4	13	67	16	0	75
3	Carrots, raw	112	5	1	59	3	32	77
4	Carrots, boiled	110	5	1	52	9	33	73
2	Mustard greens, boiled	68	5	18	61	16	0	69
5	Potatoes, sweet, baked	49	16	0	68	16	0	74
2	Pumpkin, canned	44	15	8	42	8	27	64
3	Spinach	61	17	17	52	14	0	59
1	Squash, cashew, boiled	8	69	2	22	3	4	26
2	Turnip greens, boiled	73	7	13	66	14	0	73

TABLE II
Average Percentage Digestibility of Carotenes

Number of tests averaged	20 μ g. per day per adult rat	β -Carotene	Neo- β -Carotene	α -Carotene	Neo- β -Carotene and β -Carotene
1	Carotene in oil	57	—	60	57
1	Apricots, canned	31	31	—	31
1	Beet greens, boiled	51	62	—	53
3	Carrots, raw	22	28	43	22
2	Carrots, boiled	8	9	45	8
1	Mustard greens, boiled	55	73	—	59
3	Potatoes, sweet, baked	30	51	—	35
2	Pumpkin, boiled	11	8	41	10
2	Spinach, canned	22	41	—	31
1	Squash, boiled	41	60	60	43
1	Turnip greens, boiled	43	61	—	46
	Average (vegetables) (10)	32	42	(4)47	34
	Average (greens) (4)	44	59	—	47
	60 μ g. per day per adult rat				
1	Carotene in oil	64	—	72	57
2	Carrots, raw	49	0	32	46
3	Carrots, boiled	25	35	39	27
3	Potatoes, sweet, baked	33	56	—	38
2	Pumpkin, canned	18	0	28	11
2	Turnip greens, canned	41	48	—	44
2	Spinach, canned	40	63	—	46
	Average (vegetables) (6)	34	34	(3)33	35

by the young rats than by the adult rats (average 32). The digestibility of the β -carotene dissolved in oil was 57%, higher than the average for the vegetables, but only a little higher than for the young rats. β -Carotene and neo- β -carotene may be converted one to the other, but usually little of such conversion took place during the digestion.

In each case the excrement of 4 to 6 rats was combined. When 60 μ g. of crude carotene was fed daily in two tests with raw carrots, the rats digested 49 and 48% of the β -carotene, and in three tests with boiled carrots, 29, 14, and 32%, respectively. In routine control work, the low result would be excluded and the two agreeing results accepted. The excellent agreement here secured did not prevail in all the work. With 20 μ g. daily, the β -carotene was digested from raw carrots to the extent of 21, 11, and 34%, and from the boiled carrots 2 and 14%. The variations in the former case may be due to conversion of β - to neo- β -

carotene, or vice versa, since when the β - and neo- β -carotenes are combined the digestibility from the raw carrots was 23, 20, and 25%. The object of this work was to ascertain the general digestibility of the carotene constituents from vegetables, rather than the exact digestibility in individual vegetables. Some of the variations may be due to differences in the rate of mechanical removal of the carotene from the intestines as explained by Shaw and Deuel.

Shaw and Deuel (6) fed carotene dissolved in oil to rats fasted 48 hours and found that only 2% remained in the gut 42 hours after feeding. They remark that the difference between the complete absorption reported in their tests and the incomplete absorption reported by other investigators may be due to the preliminary fasting period that their rats were subjected to. In other words, the rats being fed throughout the experiment, the continuous intake of food may have carried carotene from the intestines before digestion and absorption were completed.

The adult rats receiving a supplement of 20 μ g. of carotene daily consumed approximately 13.1 g. of basal diet per day. The rats, therefore, received the equivalent of a diet containing 1.5 μ g. of carotene per gram of ration. Similarly, the adult rats receiving 60 μ g. of carotene as supplement ate approximately 12.3 g. of the basal diet and the young rats (1) receiving the same amount of carotene supplement ate around 5.6 g. per day. These two groups therefore received diets equivalent to 4.9 and 9.1 μ g. of carotene per g. of basal diet respectively. The greater amount of carotene ingested by the young rats in proportion to the amount of basal diet eaten may have something to do with the higher average digestibility of β -carotene 52%, compared with 32% by the older rats. It is possible, despite this fact, that the younger rats have a higher digestive power for carotene because of their need both for growth and maintenance. The older rats required the carotene chiefly for maintenance.

SUMMARY

The digestibility of the β -carotene in 17 tests with adult rats weighing about 300 g. and receiving 20 μ g. of crude carotene per day ranged from 8% with boiled carrots to 55% with boiled mustard greens, and averaged 32%. In 14 tests when the rats received 60 μ g. crude carotene per day, the digestibility of the β -carotene ranged from 18% with canned pumpkins to 49% with raw carrots, and averaged 34%. The

digestion of the α -carotene averaged 47% with rats receiving 20 μ g. crude carotene per day and 33% for those receiving 60 μ g. per day. The digestibility of the neo- β -carotene B was practically the same as that of the β -carotene. The average digestibility of the carotene in the 4 greens was higher (44%) than in the other vegetables, especially carrots and pumpkins.

The digestibility of β -carotene and α -carotene dissolved in oil was about 57 and 64%, and was appreciably higher than that of the average of the carotene in vegetables (32 to 34%).

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Antibacterial Activity of Some Phenazine and Quinoxaline-Derivatives

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and

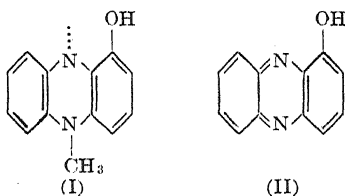
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INTRODUCTION

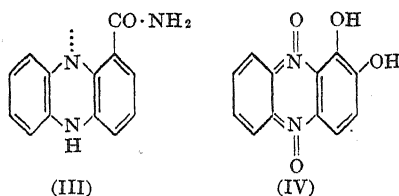
It has been known for a long time that extracts of *Pseudomonas pyocyanea* cultures inhibit bacterial growth. While it was assumed at first that a bacteriolytic enzyme "pyocyanase" was responsible for this effect, it became evident later that there are three chloroform-soluble antibacterial agents contained in such extracts (1-3).

Two of these substances have been synthesized (4, 5): the semiquinone pyocyanine (I) and hemipyocyanine or α -hydroxy-phenazine (II).



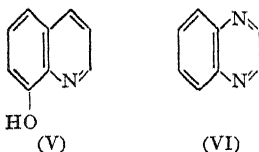
Pyocyanine is an unstable and very toxic substance and its antibiotic effect may be due to its ability to inhibit succinodehydrogenase (6). Hemipyocyanine is less toxic, non-irritating, and it inhibits the growth of streptococci in a concentration of 5 mg./100 ml. (1). Stokes, Peck, and Woodward (7) reported that α -hydroxyphenazine, while it is only about 1/5-1/10 as active against bacteria as is pyocyanine, has excellent *fungistatic* properties, much superior to those of pyocyanine.

There are two more phenazine derivatives of biological origin known to prevent bacterial growth: chlororaphin (III) from *Bacillus chlororaphis*, which is particularly active against *Streptococcus hemolyticus*, and iodinine (IV) from *Chromobacterium iodinum* which inhibited the growth of some pathogenic bacteria in a concentration of about 0.1 mg./100 ml. (8).



The possibilities of finding good bacteriostatic agents among *synthetic* phenazine derivatives have not been fully explored. A series of amino compounds was tested in 1922 and found to have relatively poor antiseptic power when compared with similar amino compounds in the acridine group (9).

It seemed interesting to us to synthesize a number of phenazine derivatives and to test their effect on bacterial growth. The question whether the complete phenazine ring system was needed for a bacteriostatic effect was of particular interest to us. The 2-ring system comparable to acridine is quinoline and it is known that some of its derivatives, such as 8-hydroxy-quinoline (V), are good antiseptics. The corresponding system containing the pyrazine ring is quinoxaline (VI), and it seemed desirable to prepare some quinoxaline derivatives and to study their effect on bacterial growth.



METHODS

1. *Synthetic Procedures.* A total of 10 phenazine and of 8 quinoxaline derivatives was tested. All compounds were synthesized in this laboratory, with the exception of P-3 and P-4 which are commercially available. References for the synthetical procedures used are given in the column following the structural formula of each compound.

TABLE I

Effect of Some Phenazine and Quinoxaline Derivatives on the Growth of Staphylococcus aureus

A. Phenazine Derivatives

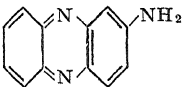
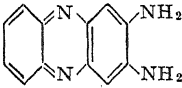
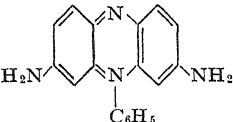
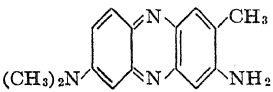
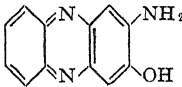
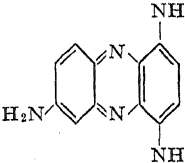
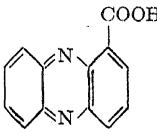
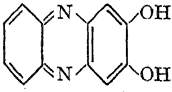
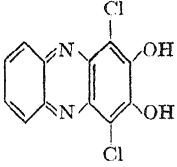
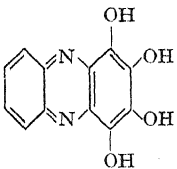
No.	Substance	Reference	Concentration mg./100 ml.	Growth of <i>Staph. aureus</i> after				
				15	21	28	72	140 hours
P-1		(13)	10 5 1 0	— + +++ +++	— ++ +++ +++	— +++ +++ +++	++ +++ +++ +++	+++ +++ +++ +++
P-2		(14)	10 5 1	+ + +++	++ ++ +++	+++ +++ +++		
P-3		—	10 5 1	— — —	— — +	+ ++ ++	++ ++ +++	++ +++ +++
P-4		—	10 5 1	— — ++	— + +++	+ ++ +++	++ +++ +++	+++ +++ +++
P-5		(14)	10 5 1	+ ++ ++	++ +++ +++	+++ +++ +++		
P-6		(15)	10 5 1	+++ +++ +++				
P-7		(11)	10 5 1	+ ++ +++	+ ++ +++	++ +++ +++	+++ +++ +++	
P-8		(16)	10 5 1	turbid, reading not possible				
				+	+	++	+++	
				+++	+++	+++	+++	

TABLE I—Continued
A. Phenazine Derivatives

No.	Substance	Reference	Concentration mg./100 ml.	Growth of <i>Staph. aureus</i> after				
				15	21	28	72	140 hours
P-9		(17)	10	—	—	—	—	—
			5	—	(+)	+	+	+
			1	++	++	++	++	++
P-10		(18)	10	—	++	++++		
			5	++	++++	++++		
			1	++	++++	++++		

B. Quinoxaline Derivatives

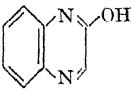
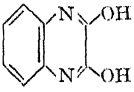
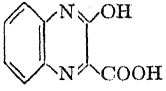
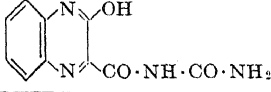
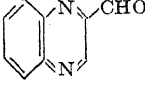
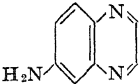
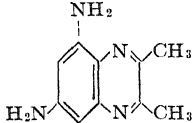
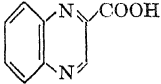
No.	Substance	Reference	Concentration mg./100 ml.	Growth of <i>Staph. aureus</i> after			
				10	21	50	140 hours
Q-1		(19)	10	+++			
			5	+++			
			1	+++			
			0	+++			
Q-2		(20)	10	+++			
			5	+++			
			1	+++			
Q-3		(19)	10	+++			
			5	+++			
			1	+++			
Q-4		(19)	10	+++			
			5	+++			
			1	+++			
Q-5		(21)	10	+	+++		
			5	++	+++		
			1	+++	+++		

TABLE I—Continued
B. Quinoxaline Derivatives

No.	Substance	Reference	Concentration mg./100 ml.	Growth of <i>Staph. aureus</i> after			
				10	21	50	140 hours
Q-6		(22)	10	++	+++		
			5	++	+++		
			1	+++	+++		
Q-7		(23)	10	(+)	+	+	+++
			5	+	++	++	+++
			1	+++	+++	+++	+++
Q-8		(21)	10	+++			
			5	+++			
			1	+++			

Numerous attempts to prepare α -phenazine carbonic acid (P-7) following the drastic procedure of Kögl (10) failed. This compound was obtained easily, however, from 1-methyl-phenazine by oxidation with chromium trioxide (11). An intermediate in the preparation of methylphenazine is 1-methyl-cyclohexane-2,3-dione, which can be obtained by treatment of dibrom-methyl-cyclohexanone with alkali. If the bromination of *o*-methyl-cyclohexanone is carried out as described by Wallach (12), serious accidents can occur. The following modified method was used instead.

Preparation of 1,3-Dibrom-1-methyl-cyclohexanone-(2). The procedure was carried out under a hood. A mixture of 22.4 g. *o*-methyl-cyclohexanone and 90 ml. glacial acetic acid in a 500 ml. Erlenmeyer flask was cooled in ice water to about 4°C. With the flask remaining in ice water, 21.8 ml. bromine were added fast (2–3 seconds) from a graduate cylinder and the windows of the hood were closed immediately. About 35–40 seconds after the first drop of bromine had been added to the methyl-cyclohexanone solution, a vigorous reaction occurred. The temperature of the mixture rose to about 60°C. and HBr escaped. Within a few seconds the mixture became pale orange to colorless and the temperature decreased rapidly. After one hour standing in ice water, the mixture was poured on ice, whereupon dibrom-methyl-cyclohexanone separated out and solidified. It was washed repeatedly with cold water, filtered by suction, and recrystallized from 65 ml. methyl alcohol. Yield: 46.5 g. (86%), m.p. 39–41°C.

2. *Bacteriostatic Tests.* The test organisms used were *Staphylococcus aureus* and *Escherichia coli*. A dilution with tryptose broth was made from 18-hour cultures, 10^{-5} for *Staph. aureus* and 10^{-6} for *E. coli*. Ten ml. of either broth were transferred to each of a number of tubes which contained water solutions (1 ml.) in various concentrations of the substances to be tested. The mixtures were incubated at 37°C.

and examined at frequent intervals for the appearance and strength of visible growth. The absence of visible growth is expressed by the symbol -, while + to +++ denote increasing amount of growth.

RESULTS

The growth of *Escherichia coli* was not inhibited or delayed by any of the 18 phenazine and quinoxaline derivatives tested in concentrations up to 10 mg./100 ml. The effect of the various compounds on the growth of *Staphylococcus aureus* is recorded in Table I.

DISCUSSION

While some of the phenazine and quinoxaline derivatives tested delayed the growth of *Staph. aureus*, none of them can be considered to be a powerful antibacterial agent. Substance P-9 (1,4-dichloro-2,3-dihydroxy-phenazine) showed the highest activity and prevented visible growth indefinitely in a concentration of 10 mg./100 ml.

As the 4 naturally occurring antibiotic phenazine compounds carry a substituent in α -position, it was interesting to see whether there is a relation between α -substitution and antibacterial action in the phenazine series. The need for a substituent in this position would be analogous to observations on anthraquinone derivatives, where only those having an α -hydroxy group can be good mordant dyes. An examination of Table I shows, however, that there is apparently no relation between α -substitution and antibiotic activity.

Replacement of the two chlorine atoms in P-9 by two hydroxy groups leads to P-10 and is accompanied by a marked decrease in antibacterial effectiveness. The compounds P-1, P-3, and P-4 do not carry a substituent in α -position, but are comparatively active.

The quinoxaline derivatives investigated here have hardly any effect on the growth of *Staph. aureus* in concentrations up to 10 mg%. A comparison of 6-amino-quinoxaline (Q-6) with 2-amino-phenazine (P-1) illustrates clearly the decrease in activity caused by the loss of one benzene ring.

SUMMARY

1. A number of phenazine and quinoxaline derivatives were synthesized and tested for bacteriostatic activity.
2. None of the 18 compounds investigated prevented or delayed the growth of *E. coli* in concentrations up to 10 mg./100 ml.

3. The growth of *Staph. aureus* was inhibited by a number of phenazine derivatives. The highest activity was shown by 1.4-dichlor-2.3-dihydroxy-phenazine, which prevented visible growth indefinitely in a concentration of 10 mg./100 ml.

4. Of 8 quinoxaline derivatives, 5 were without effect on the growth of *Staph. aureus*, while the remaining three showed a slight delaying action.

5. A comparison of 6-amino-quinoxaline with 2-amino-phenazine illustrates clearly the decrease of activity caused by the loss of one aromatic ring.

6. In the phenazine series, there is apparently no relation between substitution in α -position and antibiotic effectiveness.

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Factors Determining the Metabolic Rate of Excised Liver Tissue *

- I. Effect of Slice Thickness and Tissue Injury on Oxygen Consumption
- II. Effect of Glycogen Content on Oxygen Consumption
- III. Effect of Temperature on Oxygen Consumption

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INTRODUCTION

Although manometric methods for the measurement of metabolic processes in excised tissues have been used for many years, different investigators have reported widely varying values for a given process in tissues from normal animals. This variability indicates that some of the factors determining the rates of these processes are not yet evident, or are not always adequately controlled. It is of considerable importance that fundamental data of a nature which would aid in reducing intra-animal variability be obtained before beginning quantitative comparisons of the metabolic rates of tissues from animals undergoing various experimental procedures. This is of particular importance in excised liver because of the varied metabolic pattern of hepatic tissue.

Some of the factors determining the metabolic rate of excised liver tissue have been clearly demonstrated. These include body weight (Weymouth, Field, Kleiber, 1942; Weymouth, *et al.*, 1944), temperature of preparation (Fuhrman and Field, 1944), and the nutritional state of the animal (Rosenthal, 1935; Welch, Irving, Best, 1935; Orr and Stickland, 1941; Brazda and Rice, 1942). Although considerable data are available to show the effects of various inorganic media and substrates on the metabolic rate of liver slices, the results obtained are

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in many cases equivocal and it is difficult to formulate definite conclusions.

The investigations to be reported in this series present data showing the effect of some of the factors mentioned above, as well as a number of additional ones, on oxygen consumption and glycolysis in excised liver tissue.

METHODS

Adult albino rats of the Slonaker-Wistar strain, weighing 145 to 250 g., were used. They were fed the stock laboratory diet consisting of dried dog and fox food, water, and occasional green-stuff.

The rats were killed by decapitation, usually between 9 and 11 in the morning. The liver was removed as rapidly as possible and transferred to a cold moist chamber for slicing (Fuhrman and Field, 1944). Slices of any desired thickness were prepared with the Martin (1942) slicer, weighed rapidly on a microtorsion balance and transferred immediately to the respirometer vessels. The suspension medium was Ringer-phosphate-glucose of the following composition: NaCl 0.12 *M*, KCl 0.0024 *M*, CaCl₂ 0.0017 *M*, MgCl₂ 0.0008 *M*, Na₂HPO₄-NaH₂PO₄ 0.01 *M*, glucose 0.011 *M*, pH 7.35. The gas phase was oxygen. Oxygen consumption was determined by the direct method of Warburg (Dixon, 1943). The time between death of the animal and the beginning of thermoequilibration was kept nearly constant at 15 minutes. Four thermostatically controlled water baths, each constant to $\pm 0.1^\circ\text{C}$., were available, so that determinations could be made simultaneously at four different temperature levels. Oxygen consumption is expressed as $\mu\text{l. O}_2$ N.T.P. per mg. initial dry weight per hour (Q_{O_2}), obtained by use of the wet weight/dry weight ratio of 3.27 (Table I). We prefer the initial rather than the final dry weight as a basis for calculation of Q_{O_2} because of the variability of the latter value resulting from imbibition of water (Sperry and Brand, 1939) and the probability of incomplete recovery of tissue from the vessel at the end of a run (*cf.* Burk, *et al.*, 1941).

Glycogen was determined by the method of Good, Kramer, and Somogyi (1933) using H₂SO₄ instead of HCl for hydrolysis according to Sjögren, *et al.* (1938). Reducing substances were determined by the method of Folin (1929) modified for use with the Klett-Summerson photoelectric colorimeter. Glycogen is expressed in terms of total reducing substance determined as glucose.

I. EFFECT OF SLICE THICKNESS AND TISSUE INJURY ON OXYGEN CONSUMPTION

In order to obtain a valid measurement of the rate of oxygen consumption of excised animal tissues the preparation must be thin enough to permit adequate diffusion of oxygen and at the same time it must be prepared in such a manner that excessive tissue damage is not produced. Low rates of oxygen consumption are found when the slices are either too thick or extremely thin.

Effect of Tissue Injury

Tissue slices of uniform thickness (0.50 mm.) were prepared from a single liver; then each was further subdivided in various ways in order to determine the extent of reduction of Q_{O_2} resulting from tissue injury. The figures below represent the means of duplicate determinations on two animals. If the Q_{O_2} of a single intact slice is taken as 100, that of slices cut transversely into 3 pieces was 99, that of slices cut into 8 pieces was 91, that of minced slices was 68. The Q_{O_2} of slices ground 2 minutes in a Hayden grinder was 17 and of slices ground in a Waring blender 11. The values for ground tissue may not be directly comparable to those for sliced tissue, since the Q_{O_2} of these preparations is in part determined by the tissue/fluid ratio (Potter and Elvehjem, 1936). The marked reduction of Q_{O_2} resulting from injury is apparent from the data.

Effect of Slice Thickness

The results of a representative experiment carried out to determine the limiting thickness of rat liver slices are given in Fig. 1. It may be

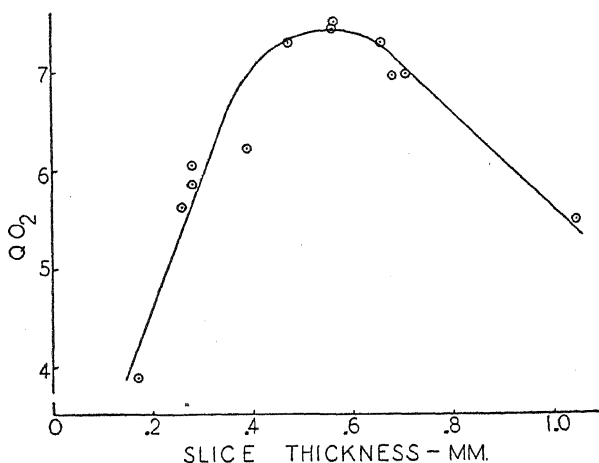


FIG. 1

Effect of Slice Thickness on the Rate of Oxygen Consumption of Rat Liver

All values were obtained using samples from a single liver. The thickness of each slice was determined before measuring the Q_{O_2} . Thickness is assumed to be equal to $\frac{\text{initial wet weight}}{\text{area}}$ without correction for specific gravity of the tissue.

seen that the maximum Q_{O_2} occurred with a slice thickness of about 0.55 mm., but that there was little difference in Q_{O_2} between 0.48 and 0.62 mm.

The limiting thickness of a tissue slice may be calculated from the formula derived by Warburg (1923):

$$d' = \sqrt{8C_o \frac{D}{A}} \quad (1)$$

in which d' = thickness in cm. at which the oxygen concentration at the center of the slice is zero; C_o = oxygen concentration in atmospheres outside the slice; D = diffusion constant; and A = rate of oxygen consumption in cc. per minute per cc. tissue.

Under the conditions used for obtaining the data in Fig. 1, $C_o = 1$, $A = 3.83 \times 10^{-2}$ (equal to a Q_{O_2} of 7.53, the highest value given in Fig. 1), and $D = 1.7 \times 10^{-5}$ at 38°C. (Krogh, 1919; cf. Hill, 1928). Using these values $d' = 0.059$ cm., a figure agreeing well with that determined experimentally (0.055 cm.).

The assumptions which were made in the above calculation, and which have been made frequently for similar calculations, may not all be valid, so that it is of importance to point out some of the sources of error. Since A varies with the experimental conditions, and is frequently lower than 5×10^{-2} , originally obtained by Barcroft and Shore (1912) and used by Warburg (1923) and Dixon (1943), it seems preferable to use a value more nearly representing the maximum obtainable under a given set of experimental conditions. This would assure a slice thin enough to permit adequate oxygen diffusion.

Since the diffusion constant has not been determined for liver, the value used is that obtained on diaphragm at 20°C. (Krogh, 1919), corrected for temperature to 38°C. (Hill, 1928). This figure is probably more nearly correct for the experimental conditions usually employed in mammalian tissue studies than is the lower value used by Warburg (1923) and Dixon (1943).

Calculation of limiting thickness from formula (1) assumes that the oxygen consumption does not decrease until the oxygen tension at the center of the slice approaches zero. In view of the results obtained on other tissues under conditions of reduced oxygen tension this assumption may not be valid (cf. Kempner, 1939; Warren, 1942; Craig and Beecher, 1943).

Although it may be that the agreement between the limiting thickness of liver slices calculated from formula (1) and determined experimentally (Fig. 1) is in part fortuitous, it is clear that under these condi-

tions the maximum rate of oxygen consumption is obtained using slices 0.5 to 0.6 mm. thick, rather than somewhat thinner slices as has been recommended (Dixon, 1943). When a series of animals is used to obtain data similar to that shown in Fig. 1, the additional factor of intra-animal variability is introduced. However, the same optimum thickness was found using liver slices from ten different animals under conditions similar to those employed here (unpublished data obtained with J. J. Lewis).

DISCUSSION

The importance of maintaining cell structure during the measurement of metabolic processes in excised tissue has been pointed out by others (Druckrey, 1935 and 1936; Fleischmann, 1936; Commoner, 1942; Shorr, 1942; Krebs, 1943; Barron, 1943), and is clearly shown by the findings that certain reactions which take place in tissue slices fail to occur in minced tissue. However, the quantitative differences in the rate of oxygen uptake between intact slices and those damaged by slicing too thin have not always been appreciated, although emphasized by Shorr (1942). The effort to obtain adequate oxygen diffusion has thus encouraged some workers to slice tissue so thin that low rates of oxygen consumption were obtained.

It is beyond the scope of the present paper to discuss the causes of the decrease in oxygen consumption in damaged tissue other than to attribute it to cell injury and destruction. If the tissue is sufficiently disintegrated the oxygen consumption is essentially determined by the activity of the resulting solution of enzymes and particulates (*cf.* de Robertis and Nowinski, 1942; Barron, 1943; Lazarow, 1943). The dilution of enzymes under these conditions compared to their concentration in the intact cells may then result in a lower rate of oxygen consumption because of the decreased probability of a ternary collision as pointed out by Krebs (1943).

II. EFFECT OF GLYCOGEN CONTENT ON OXYGEN CONSUMPTION

It was observed in the course of this investigation that the Q_{O_2} of liver from starved rats was higher, on either a dry weight or wet weight basis, than the Q_{O_2} of liver from well fed animals. Walthard (1934) reported that the Q_{O_2} of rat liver is lowered by high glycogen content, and attributed the decrease to regressive tissue changes. Since

glycogen is the liver component which varies most readily with the nutritional state of the animal, experiments were carried out to examine the relationship between Q_{O_2} and the glycogen content of rat liver.

Low liver glycogen values were obtained by fasting the rats for periods varying from 12 to 24 hours. High glycogen contents were obtained by feeding a diet consisting of 50% dry dog food and 50% glucose for varying periods up to 24 hours, following a 24 hour fast.

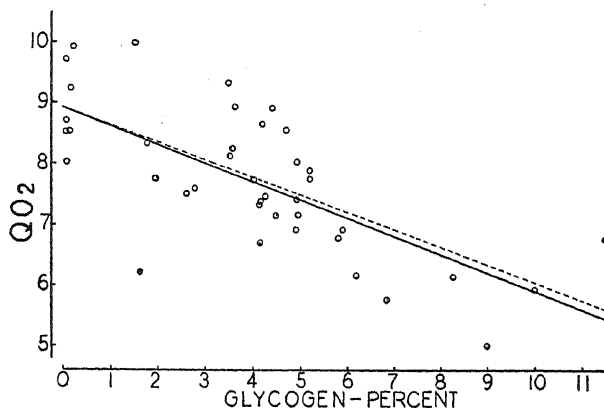


FIG. 2

Graph Showing the Rate of Oxygen Consumption of Rat Liver Slices as a Function of Glycogen Content

Each point represents the mean of duplicate determinations of both Q_{O_2} and glycogen. The solid line is fitted to the data by the method of least squares. The dotted line is drawn to represent the theoretical relationship as explained in text.

Water was available at all times. No determinations were made on livers from animals in which high glycogen was maintained for periods longer than 24 hours.

In Fig. 2 the data are presented in the form of a graph showing Q_{O_2} as a function of per cent liver glycogen. The data were obtained on 40 rats in which liver glycogen ranged from 0.03 to 11.5% (g. per 100 g. wet weight). It may be seen that the Q_{O_2} decreases with increasing glycogen content of the liver. The solid line drawn through the points in Fig. 2 was fitted by the method of least squares. The correlation between Q_{O_2} and per cent glycogen is 0.708. The solid line drawn may

be represented by the equation

$$Q_{O_2}GF = Q_{O_2} - (-0.3046G) \quad (2)$$

in which $Q_{O_2}GF$ is the Q_{O_2} calculated on a glycogen-free basis, Q_{O_2} is the experimentally determined rate of oxygen consumption, and G is the liver glycogen in per cent.

The relationship shown in Fig. 2 is not the consequence of changes in water content coincidental with changes in glycogen content. The conversion of Q_{O_2} determined on a wet weight basis to Q_{O_2} on a dry weight basis (Fig. 2) was made by means of the W/D ratio determined from the water content of the livers from 42 animals (Table I). No

TABLE I

Water Content of Rat Liver

Liver samples taken immediately after death by decapitation and exsanguination.

Dried to constant weight at 105°C. All determinations made in duplicate.

Number of animals	42
Mean percentage water	69.44
Standard deviation	1.218
Standard error of mean	0.190
Mean W/D Ratio*	3.27

* W/D Ratio = Wet weight/Dry weight. The Q_{O_2} on a wet weight basis may be converted to Q_{O_2} on a dry weight basis by multiplication by this factor.

correlation was found between water and glycogen content of the liver in this series. Even though the glycogen content varies widely, the water content remains constant, indicating that in the rat, liver glycogen is deposited with approximately the same amount of water as is associated with the other liver solids (Fenn, 1939). For the series of animals used here (Table I) it was found that the mean water content of the liver was 69.44%. Each gram of dry liver thus had 2.27 ml. of water associated with it.

A theoretical relationship between Q_{O_2} and liver glycogen similar to that shown in Fig. 2 may be derived if it be assumed that glycogen is a stored component of the liver and that, if sufficient substrate is present, it does not contribute to the rate of oxygen consumption. Since glucose was present in the medium in all experiments, ample substrate was available. If the liver slice is regarded as active tissue diluted with glycogen and water, the observed Q_{O_2} may be corrected for the presence of an inert mass composed of the glycogen and accompanying water.

In Fig. 2 the dotted line is drawn to represent this theoretical relationship, assuming that the $Q_{O_2}GF$ is 8.934 (the y intercept of the solid line fitted to the experimental data) and that 1 g. of glycogen is deposited in the liver together with 2.27 ml. of water. This relationship may be represented by the equation

$$Q_{O_2}GF = Q_{O_2} - (-0.2922G) \quad (3)$$

in which the symbols are the same as those used in equation (2). The slope of this line is statistically not significantly different from the slope of the line fitted to the experimental data.

It may be seen from Fig. 2 that the liver glycogen content is an important factor in determining the rate of oxygen consumption of excised liver. For comparison of rates of oxygen consumption between different groups of animals it is essential that the observed Q_{O_2} be corrected for the glycogen content of the liver from which the sample was taken. This is most easily done by reducing all values to a glycogen-free basis ($Q_{O_2}GF$). Since the slope of the line represented by equation (3) varies with the temperature and is changed if Q_{O_2} is expressed on a wet rather than a dry weight basis, the following relationship was derived for the conversion of observed Q_{O_2} to glycogen-free Q_{O_2} ($Q_{O_2}GF$):

$$\frac{100}{100 - (3.27G)} \times Q_{O_2} = Q_{O_2}GF \quad (4)$$

The symbols are the same as those previously used, and 3.27 is the weight of 1 g. of glycogen plus the water associated with it as calculated from the data given in Table I. This correction is valid at all temperatures and may be applied to Q_{O_2} expressed on either a wet or dry weight basis.

DISCUSSION

Craig (1943) recently reported the Q_{O_2} of liver from fed rats to be 7.3 and that from fasted rats to be 8.2. Assuming that these values would be identical on a glycogen-free basis, the glycogen content of the fed rat liver necessary to obtain the observed Q_{O_2} may be calculated. The glycogen content of the livers of fasted rats after 24 hours has been reported as about 0.2% (Fenn, 1939; Conant, *et al.*, 1941), so that the Q_{O_2} of 8.2 would become 8.3 on a glycogen-free basis (Equation 4). A $Q_{O_2}GF$ of 8.3 for the liver from the fed animals would, ac-

according to equation (4), be obtained when the glycogen content was 3.7%. This value is in good agreement with the values reported in the literature for the glycogen content of the liver of fed rats (3.8%—Fenn, 1939; 4.0%—Newburger and Brown, 1942).

Since the values plotted in Fig. 2 show considerable variability that is not accounted for on the basis of differences in glycogen content it seems probable that varying amounts of other "inert" components may be involved. Welch, Irving, and Best (1935) reported decreases in Q_{O_2} of the liver associated with increased fat, but the decreases in oxygen consumption were greater than could be accounted for by dilution of the active protoplasm with fat (Best, *et al.*, 1936; *cf.* Ennor, 1942).

III. EFFECT OF TEMPERATURE ON OXYGEN CONSUMPTION

The oxygen consumption of excised liver slices has not previously been determined over a wide range of experimental temperatures. Data of this nature are of interest for comparison with the effects of temperature on other tissues (*cf.* Field, Fuhrman, Martin, 1944; Field and Hall, 1944; Fuhrman and Field, 1942).

The rate of oxygen consumption of rat liver slices at graded temperature levels from 0.2° to 45°C. is shown in Fig. 3. The liver slices used for these determinations were taken from normal fed animals, and the Q_{O_2} on a glycogen-free basis ($Q_{O_2}GF$) calculated from the observed Q_{O_2} and liver glycogen content according to equation (4).

Constancy of Q_{O_2} with Time

At 37.7°C. the rate of oxygen consumption was constant with time for at least three hours, and for four hours in some experiments. At temperatures below 37.7°C. no decrease in Q_{O_2} with time was observed, although in most instances these experiments were continued for only one to two hours. At 42° and 45°C. Q_{O_2} was constant with time for only 30 to 60 minutes.

In these experiments the rates of oxygen consumption used for the construction of Fig. 3 were calculated for the first 30 minutes after thermoequilibration. If later time periods were used for the calculation of Q_{O_2} at these high temperatures the rates of oxygen consumption at 42° and 45°C. would be lower than those shown in Fig. 3. No attempt was made to determine accurately the optimum temperature for

oxygen consumption of liver under these conditions, but it is clear that this optimum is above 37.7°C. The decline in Q_{O_2} with time associated with supra-normal experimental temperatures has been discussed more fully by Field, Fuhrman, and Martin (1944).

Oxygen Consumption at Graded Temperature Levels

It may be seen from Fig. 3 that for the temperature range 25° to 45°C., $Q_{O_2}GF$ is an approximately linear function of temperature in degrees Centigrade. Below 15°C. there is also an approximately linear

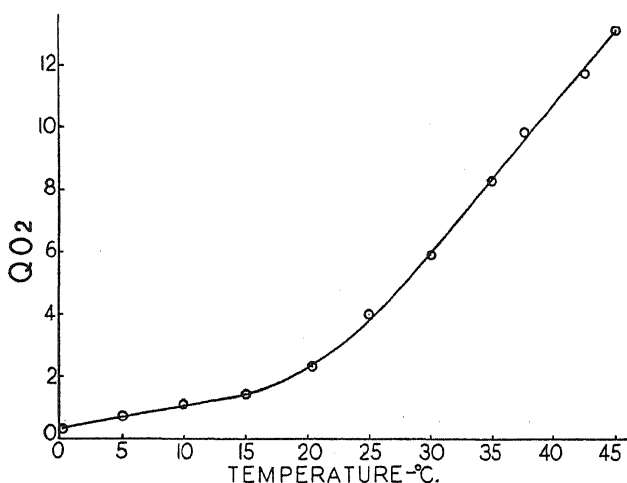


FIG. 3

Graph Showing the Rate of Oxygen Consumption of Rat Liver Slices as a Function of Temperature

Each point represents the mean of at least four determinations. Oxygen consumption rate is expressed on a glycogen-free basis ($Q_{O_2}GF$).

segment of the curve. The general shape of this curve is considerably different from that obtained for cerebral cortex (Field, Fuhrman, Martin, 1944), kidney cortex (Fuhrman and Field, 1942) or heart (G. J. Fuhrman and Field, unpublished).

Since a graph of $Q_{O_2}GF$ as a function of temperature results in a curve consisting of two linear segments, it is unlikely that further mathematical analysis of the data by customary methods (*cf.* Belehrad, 1935) would greatly clarify the processes underlying such a rela-

tionship. For purposes of comparison with other data, however, two temperature coefficients have been calculated. Over the range 15° to 30°C. the mean value of the van't Hoff coefficient, Q_{10} , is 2.56, while over the range 5° to 35°C. the mean value of the Arrhenius coefficient, μ , is 13,850. Both of these temperature coefficients vary with the temperature over the ranges given.

Reversibility of Inhibition Produced by Cold

It has previously been found for rat cerebral cortex and kidney cortex slices that the inhibition of oxygen consumption produced by maintenance of the tissue for one hour at 0.2°C. was completely reversible when the tissue was rewarmed to 37.7°C. (Fuhrman and Field, 1943). Similar experiments were carried out with liver slices. The rate of oxygen consumption at 0.2°C. was not determined in these experiments, but in others the Q_{O_2} GF at that temperature was found to be 0.28, indicating an inhibition of about 96.5% produced by the low temperature. Using duplicate samples of liver from two rats the initial Q_{O_2} at 37.7°C. was 7.89, and after 1 and 2 hours at 0.2°C. the Q_{O_2} was 7.70 and 7.79, respectively. These differences are within the limits of error of the method. Thus with liver, as with cerebral cortex and kidney cortex, the inhibition of oxygen consumption produced by maintenance of the tissue at 0.2°C. for one hour is completely reversible.

SUMMARY

1. The optimum thickness for oxygen consumption in rat liver slices at 37.7°C. was determined experimentally to be about 0.55 mm., and calculated (using Warburg's formula) to be 0.59 mm. Lower rates of oxygen consumption were obtained with slices either thicker or thinner than 0.5–0.6 mm. Tissue injury produced by further subdivision of the 0.5 mm. slices resulted in lower rates of oxygen consumption.

2. The rate of oxygen consumption of rat liver slices was found to vary inversely as the glycogen content. This relationship could be satisfactorily explained by the hypothesis that the active liver tissue was diluted with glycogen and water deposited with it. Variability in the rate of oxygen consumption of liver may be reduced by expressing the Q_{O_2} on a glycogen-free basis. A method for making this correction is given.

3. The rate of oxygen consumption of rat liver slices was deter-

mined at graded temperature levels from 0.2° to 45°C. Neither Q_{10} nor μ was constant over any considerable range of temperature. The inhibition of oxygen consumption produced by maintaining liver tissue for 1 or 2 hours at 0.2°C. was found to be completely reversible.

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Purification and Properties of Arachain, a Newly Discovered Proteolytic Enzyme of the Peanut

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INTRODUCTION

The meal that remains after the removal of the oil from raw peanuts by solvent extraction contains approximately 50 per cent protein. Buffered suspensions of such a meal, when protected against microbiological activity, autolyze at a moderate rate, whereas similar suspensions of commercial peanut meal, from which the oil has been removed by cooking and hydraulic pressing, exhibit no appreciable autolytic activity. This difference in behavior points to the presence in the peanut of heat labile proteolytic enzymes. Information concerning the properties of the proteolytic enzymes of the peanut is lacking; yet, in addition to its academic interest, such information is of practical value in the use of peanut flours as food ingredients (1) and in the processing of peanut meal for protein.

We have found in peanut meal a proteolytic enzyme that hydrolyzes benzoyl-*L*-arginine amide rapidly in the absence of added activators. It is our purpose to describe in this paper the preparation of potent concentrates of this enzyme, for which the name arachain is suggested, and to present data concerning its pH optimum, thermal stability, and the kinetics of its action on benzoyl-*L*-arginine amide.

EXPERIMENTAL

Materials and Methods

Benzoyl-*L*-arginine amide (BAA) was prepared as described by Bergmann, Fruton, and Pollock (2). For the enzyme experiments an aqueous solution containing 0.125

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mM. of the substrate per ml. was prepared and protected against spoilage by means of a few crystals of thymol. Hydrolyses were carried out in Pyrex, glass-stoppered, 2.5 ml. volumetric flasks. The test solution in each case had the following composition: 1.0 ml. of substrate solution; 0.5 ml. of 0.1 M phosphate-citrate buffer (3) of the pH desired; the meal or enzyme solution in the amount desired; and water to bring the volume of 2.5 ml. The extent of hydrolysis was determined on 0.2 ml. samples at intervals during incubation by the microtitration method of Grassmann and Heyde (4). The pH determinations were made by means of a glass electrode.

*Action of Whole Peanut Meal, Various Parts of the Peanut, and Meal from
Germinated Peanuts on Benzoyl-L-arginine Amide*

The action of these preparations on BAA at pH 6.0 and 40°C. is shown in Table I, together with comparative data for soybean and cottonseed meals. The samples were prepared in the following manner. Red-skinned peanuts were hand-shelled and 100 g.

TABLE I

*Action of Peanut Meal, Various Parts of the Peanut, and Germinated Peanut Meal
on Benzoyl-L-arginine Amide*

40 mg. meal per ml. test solution; pH 6.03-6.25; duration of hydrolysis,
60 minutes; temperature, 40°C.

Sample designation	Description	Total nitrogen ¹ per cent	Extent of hydrolysis ² per cent	Hydrolysis per mg. of meal
				nitrogen ³ per cent
64G	Whole-peanut meal ⁴	8.90	31	8.7
65G	Whole-peanut meal	9.15	31	8.5
66G	Peanut cotyledons	9.17	34	9.3
67G	Peanut skins	2.44	0	0.0
68G	Peanut germs	7.97	16	5.0
69G	Germinated peanuts, 48 hrs.	8.71	32	9.2
70G	Germinated peanuts, 72 hrs.	8.58	34	9.9
71G	Germinated peanuts, 96 hrs.	8.14	32	9.8
12F	Soybean meal ⁴	8.10	8	2.5
21F	Cottonseed meal ⁴	8.24	10	3.0

¹ "As is" basis.

² Corrected for slight autolysis of the meal (not more than 2 per cent) in the absence of substrate.

³ Per cent hydrolysis/40 × per cent total nitrogen.

⁴ Petroleum ether-extracted; all others acetone-extracted.

of the whole kernels (including the skins and germs) were extracted in a Waring Blendor at room temperature with three successive 150 ml. portions of petroleum ether to obtain Sample 64G. Another 100 g. portion of the whole kernels was extracted similarly with acetone to obtain Sample 65G. A third 100 g. portion of the whole kernels was separated by hand into cotyledons, skins, and germs, and each of the

three lots was extracted with three changes of acetone to obtain Samples 66G, 67G and 68G, respectively. Three samples of the whole kernels (each consisting of 41 kernels and weighing 25 g.) were allowed to germinate at 28.5°C.—one for 48 hours, another for 72 hours, and the third for 96 hours; there was 98 per cent germination in each case. The germinated nuts were extracted with three changes of acetone to obtain Samples 69G, 70G, 71G, respectively. The samples of soybean and cottonseed meal were prepared by extracting the flaked seeds with petroleum ether in a Soxhlet extractor. All of the extracted samples were air-dried and ground in a small-sized Wiley mill to pass a 60-mesh sieve.

TABLE II
*Proportions of Cotyledons, Germs, and Skins in the Peanut Kernel, and
Ash and Nitrogen Contents of the Whole Kernel and Oil-free Parts*

Sample	Fraction of whole peanut kernel per cent	Ash ¹ per cent	Total nitrogen ² per cent
Whole kernel	100	5.75	10.55
Cotyledons	95.5	4.65	10.60
Germs	2.0	6.65	9.46
Skins	2.5	3.08	2.84

¹ Oil-free, dry basis.

² Oil-free, dry, ash-free basis.

It is evident from Table I that peanut meal contains an enzyme that causes rapid hydrolysis of BAA to yield benzoyl-L-arginine and ammonia. This enzyme is present in the cotyledons and to a lesser extent in the germ, but it is absent from the skin. Two meals obtained from whole peanut kernels by extracting in one case with petroleum ether and in the other with acetone were equally potent enzymatically. Germination for 48 or 72 hours resulted in a significant, but not a marked, increase in the amount of enzyme present; no further increase appeared to result from continuing the germination for another 24 hours.

Both soybean meal and cottonseed meal appear to contain considerably less of this type of enzyme than does peanut meal, but since the amount of active enzyme in a seed meal would undoubtedly depend on the age of the meal and the factors involved in preparation, comparisons based on few data should be made with caution. Recently Laufer, Tauber, and Davis (5) recognized the presence in the soybean of a proteolytic enzyme system that hydrolyzes gelatin and casein with a pH optimum in the range of 6.5–7.0 and for which they proposed the name soysin. No attempt was made to prepare concentrates

of the enzyme or to study its activation properties, but it was observed that the amount of enzyme is doubled after 6 days of germination and increases still further when germination is continued for another 6 days.

A few data concerning the specificity of the enzyme contained in peanut meal have been obtained. Petroleum ether-extracted peanut meal, in the same amounts used for the tests reported in Table I and under identical test conditions with regard to substrate concentration, temperature and pH, is completely inactive toward the following substrates: carbobenzoxyglycylphenylalanine, carbobenzoxyglycyltyrosine, and carbobenzoxyglutamyltyrosine.

A partial analysis of the various parts of the peanut kernel and the proportion of each part in the kernel are shown in Table II. The rather high ash content of the germ, as compared with that of other parts of the kernel, is noteworthy. However, spectrographic examination of the ash from each of the parts revealed no qualitative differences between them.

Purification of Arachain

Preliminary attempts to separate arachain from peanut meal indicated that the solutions obtained by extracting the meal at pH values between 4.5 and 7.0 were almost completely inactive toward BAA. Inasmuch as the bulk of the protein of peanut meal is extracted by dilute alkali at pH 7.0 (6), and all of the extracts prepared from the meal (including that made at pH 7.0) were nearly devoid of activity, it is apparent that the enzyme, if not destroyed, must remain in the extracted meal residue. Semiquantitative tests made on the extracted residue revealed that a large part of the enzymatic activity was present in this material, and that substantial amounts of the enzyme could be removed by extraction with dilute sodium chloride solution.

On the basis of these observations, the following scheme was devised for separating arachain from peanut meal. Ten grams of petroleum ether-extracted peanut meal (Sample 26F)¹ was stirred for 15 minutes at room temperature with 100 ml. of water and sufficient *N* sodium hydroxide to bring the suspension to pH 6.9. The suspension was centrifuged; the supernatant solution (A) was removed, and the residue was extracted with two successive 50-ml. portions of water. The wash solutions (pH 7.4) were combined (B) and the meal residue was stirred with 100 ml. of 0.5 *N* sodium chloride for 45 minutes at 4°C. (pH 6.6). The extract (C) was removed by centrifuging, and the residue was reextracted in the cold with two successive 50-ml. portions

¹ This sample of meal was prepared from a special variety of peanuts having white skins (6, 7).

of the salt solution to yield the combined extract (D). Extract (C) was dialyzed against running distilled water in the cold for 20 hours, and the supernatant solution (E) and the precipitate (F), which formed on dialysis, were separated by centrifugation. One ml. of each of the solutions obtained was tested for activity toward BAA.

TABLE III

Occurrence of Arachain Activity and Distribution of Total Nitrogen in Various Fractions Obtained from Peanut Meal

Substrate, benzoyl-L-arginine amide; pH 6.0; temperature, 40°C.

Sample designation	Description	Amount used per ml. test solution ¹	Duration of hydrolysis	Extent of hydrolysis ²	Enzyme potency ³	Total activity		Total nitrogen
		mg. N	min.	per cent	units/mg. N	units	per cent	per cent
26F	Peanut meal, 10 g.	3.6	60	31	0.58	527	100	100
A	Aqueous extract, pH 6.9	3.3	1100	69	0.083 ⁴	60	11	79.5
B	Aqueous wash, pH 7.4	0.46	1100	23	0.18 ⁴	20	4	12.4
C	Sodium chloride extract, pH 6.6	0.10	60	12	8.0	200	38	2.8
D	Sodium chloride wash, pH 6.8	0.029	150	12	11.0	74	14	0.7
						—	—	—
						354	67	95.4
E	Solution after dialysis of (C)	0.0092	300	0	0.0	0	0	0.3
F	Precipitate after dialysis of (C)	0.15	94	31	8.8	158	32	2.0
G	Sodium chloride extract of (F)	0.13	94	30	9.8	156	31	1.7

¹ All figures refer to total nitrogen. In the meal and in fraction A, approximately 6 per cent of the total nitrogen is non-protein; in the other fractions, all but a trace is protein nitrogen.

² Corrected for slight autolysis in absence of substrate. In all cases except fractions A and B, increase in titration due to autolysis varied from 0 to 2 per cent; in the case of fractions A and B, autolytic activity amounting to approximately 4 per cent was observed.

³ Enzyme unit = amount of enzyme required to hydrolyze 0.25 per cent of substrate per minute.

⁴ These are minimum values due to the fact that the period of hydrolysis required for assay is probably long enough to permit appreciable destruction of enzyme at this temperature.

In the case of the precipitate (F) obtained on dialysis of the salt extract (C) of the meal residue, a suspension in 0.5 *N* sodium chloride was prepared, and 1 ml. of the suspension was used for testing. Since all of the precipitate failed to dissolve under these conditions, the suspension was centrifuged to yield a clear solution (G), 1 ml. of which was also tested for enzymatic activity. Total nitrogen was also determined on a portion of each solution. The results of the enzyme tests, together with data on the distribution of the meal nitrogen in the several fractions, are given in Table III. Data for the peanut meal used as the starting material are also included in the table.

For the results reported in Table III and for those of all subsequent experiments, the arachain unit was considered to be the amount of enzyme required to hydrolyze BAA at the rate of 0.25 per cent per minute under the conditions of the test. The choice of the fraction of substrate hydrolyzed per unit time as a measure of enzyme activity is valid in view of the kinetics of this enzyme reaction, which will be described in a later section. It should be pointed out, however, that the potency of a given preparation, as expressed in terms of the unit described, will depend upon the temperature at which the test is made, since it is obvious that the rate of hydrolysis will be higher for higher temperatures. Inasmuch as some of the experiments were conducted at 40°C., while others were conducted at 30°C., the potency of all preparations is expressed in terms of units per mg. of nitrogen, or per ml. of enzyme solution, at the temperature used in the test.

The data indicate that, as a result of the fractionation, approximately 50 per cent of the original meal's activity was in the potent fractions C and D which, together, comprised only 3.5 per cent of the original meal's nitrogen. Consequently, the fractionation resulted in a 14- to 19-fold increase in the potency of the enzyme. The two initial steps in the fractionation procedure accounted for the major part of the purification, since the extraction and washing with dilute alkali at pH 6.9 to 7.4 removed 92 per cent of the meal nitrogen but only 15 per cent of the total arachain activity. Apparently, the first sodium chloride extract (C) also contained some inert protein, since the potency of this fraction was appreciably less than that of the second sodium chloride extract (D).

Approximately 35 per cent of the meal's activity was unaccounted for after the fractionation process, but the 65-per cent recovery might be considered excellent in view of the extreme sensitivity of the enzyme to thermal destruction and the fact that the assays for at least two of the fractions (A and B of Table III) were probably minimum values.

In the case of these two fractions the period of hydrolysis required for assay was probably long enough to permit appreciable destruction of the enzyme at the temperature used. Furthermore, qualitative tests revealed that a small amount of activity remained in the extracted meal residue.

Approximately 15 per cent of the activity of the sodium chloride extract (C) was lost during dialysis against distilled water. Since this step resulted in only a slight increase in potency, and since the enzyme protein was insoluble in water and had to be redissolved in sodium chloride solution for use, little advantage was gained by dialysis against

TABLE IV

Effect of Various Substances on the Activity of Arachain

Substrate, benzoyl-L-arginine amide; enzyme concentration, 0.2 ml. per ml. test solution; duration of hydrolysis, 60 min.; temperature, 40°C.; pH 6.0.

Substance ¹ added	Extent of hydrolysis per cent
None	12
Cysteine	12
Ascorbic acid	13
Hydrogen cyanide	12
Hydrogen peroxide	10

¹ Concentration, 0.02 *mM* per ml. test solution.

distilled water. The experience gained in several fractionation experiments similar to that described, has shown that dialysis of the 0.5 *N* sodium chloride extract against 1-per cent sodium chloride in the cold results in no appreciable destruction of the enzyme and yields a clear solution that contains only 1.8 per cent of the meal nitrogen and has a potency of approximately 12 units per mg. of nitrogen at 40°C. Many attempts to increase the potency of the enzyme preparations beyond this value have been unsuccessful.

Effect of Several Substances on the Activity of Arachain: Portions of a sample of purified arachain solution were treated with cysteine, ascorbic acid, hydrogen cyanide, and hydrogen peroxide to determine if any of these substances would affect the rate of hydrolysis of BAA by the enzyme. Each of the substances employed was added to the enzyme in the form of 0.1 *M* solution, which had been freshly prepared and adjusted to approximately pH 6.0 immediately before use. The results are summarized in Table IV.

Neither of the two substances (cysteine and hydrogen cyanide) that are commonly used for the activation of enzymes of the papain type had any activating effect on arachain. Likewise, hydrogen peroxide, which inhibits enzymes of the papain type, had no appreciable effect on the activity of arachain. Moreover, it was found that the meal itself contained no enzyme that was activated by cysteine for the hydrolysis of BAA at pH 5.5–6.0, and that the rate of autolysis of peanut meal, in the absence of added substrate was not increased by the addition of cysteine. It may be concluded from these observations that neither arachain nor any of the other possible proteolytic enzymes of the peanut belong to the group of plant enzymes that have been designated "papainases" and include, besides papain (8), such enzymes as ficin from the fig (9), bromelin from the pineapple (10, 11), a proteinase of wheat flour (12), and the asclepains from milkweed (11).

Arachain appears to resemble more closely solanain from the horse nettle (11) and hurain from the sap of the Venezuelan tree *Hura crepitans* (13). Since the properties and enzymatic behavior of both solanain and hurain have been likened to those of trypsin (11, 13), it is noteworthy that benzoyl-L-arginine amide, which is split rapidly by arachain, is also a typical substrate for trypsin, and that like trypsin, arachain apparently requires no activator. This behavior differs from that of the papainases, which also hydrolyze benzoyl-L-arginine amide but in all cases require an activator for the development of maximum activity. These observations may provide additional justification for considering the suggestion of Jaffe (13) that a new group of plant enzymes be established to include those that are not activated and possess other characteristics distinct from those of the papainases. Based on the information now available this new group would include hurain, solanain, and arachain.²

pH Optimum of Arachain

Experiments to determine the influence of pH on the activity of arachain were carried out at 30°C., a purified sample of arachain being used. McIlvane (0.1 M) phosphate-citrate buffers (3) were used throughout. The results are presented graphically in Fig 1.

² The name *arachain* has been proposed for this enzyme in accordance with the suggestion of Lineweaver [mentioned in the paper of Greenberg and Winnick (11)] that appropriate names with the suffix "ain" be used for the designation of plant proteases.

The pH optimum for arachain is rather broad, and covers the pH range 6.0–7.5 or above. Inasmuch as several of the assays reported in this paper were conducted at pH 6.0, it is fortunate that, according to Fig. 1, such assays should yield values only slightly lower than those performed at pH 7.0.

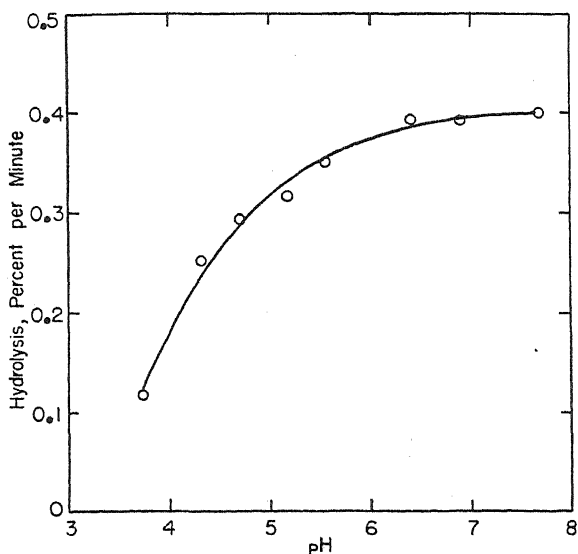


FIG. 1

Influence of pH on the Activity of Arachain
Substrate, benzoyl-*L*-arginine amide; temperature, 30°C.

Kinetics of the Action of Arachain on Benzoyl-L-arginine Amide

Because it had been previously demonstrated that the kinetics of the hydrolytic action of a large number of proteolytic enzymes on dilute solutions of synthetic substrates follow the equation for a first-order reaction, an attempt was made to determine if the action of arachain on BAA is also of the first order. Among the enzymes that exhibit first-order-reaction kinetics are papain and ficin (14), trypsin (15), cathepsins I, II, III, IV (16, 17), certain of the intestinal peptidases (18), carboxypeptidase (20), and chymotrypsin (19). It was soon found that the kinetics of the reaction of arachain with BAA deviate appreciably from those characteristic of a first-order reaction. This is

evident in Table V where first-order reaction "constants" have been calculated for typical data obtained in the hydrolysis of BAA by several concentrations of arachain. It is obvious that a first-order reaction constant is not obtained in any case, since the calculated K values increase progressively as the duration of hydrolysis increases.

In his discussion of the kinetics of hydrolytic enzymes, Van Slyke (21) points out that the enzymatic process is characteristically a two-stage reaction. In the first stage the enzyme, in the presence of a high concentration of substrate, is saturated with substrate, and the time required to effect substrate-enzyme combination is at an irreducible minimum. Consequently, the rate-determining factor for the hydrolysis is the speed with which the substrate is decomposed. As long as the substrate concentration remains sufficiently high to completely saturate the enzyme, the reaction continues in the first stage, the reaction proceeds at maximal speed, and the amount of substrate split per unit time is constant. This zero-order velocity constant (K_D = fraction of substrate split/time) is a measure of the velocity of decomposition of the substrate and is directly proportional to the enzyme concentration. The second stage of the enzymatic reaction begins when the substrate concentration is reduced to such a level that the enzyme is no longer saturated with substrate. Under these conditions, the rate of the reaction is determined by the rate with which substrate and enzyme combine, and the process imitates a monomolecular reaction. The first-order velocity constant for the second stage of the reaction ($K_C = 1/t \log a/a - x$) is a measure of the rate of combination of enzyme and substrate and is also directly proportional to the enzyme concentration.

When the hydrolysis values given in Table V are plotted against time, a straight line is obtained, which indicates that a constant amount of substrate is split during each time interval. The reaction between arachain and BAA appears to conform, therefore, to the first stage of the two-stage reaction described above. The calculated values for K_D (Table V) for each concentration of enzyme used are practically constant over the measured course of the hydrolysis, and the average K_D values are directly proportional to the enzyme concentration, as is evident from the constancy of the values given for the proteolytic coefficient (last column of Table V). There is no indication from the data of a tendency for the K_D values to diminish, even after the substrate concentration is reduced to one-tenth of its initial value. In other words, there appears to be no indication that substrate concentrations sufficiently low to permit the establishment of the second stage of the enzymatic reaction have been approached in these experiments. It must be concluded, therefore, that the enzyme is still saturated with substrate even at extremely low substrate concentrations (0.05–0.005

TABLE V

Kinetics of the Reaction of Arachain and Benzoyl-L-arginine Amide

Enzyme concentration ml./ml. test sol.	Duration of hydrolysis min.	Extent of hydrolysis per cent	K_c^1	K_D^2	Average K_D	C_D^3
0.20	40	8	0.0009	0.20	0.21	1.05
	80	18	0.0011	0.22		
	203	42	0.0012	0.21		
	300	64	0.0015	0.21		
0.30	40	10	0.0012	(0.25)	0.30	1.00
	80	23	0.0014	0.29		
	202	60	0.0020	0.30		
	300	90	0.0033	0.30		
0.40	40	17	0.0020	0.42	0.42	1.05
	80	34	0.0023	0.42		
	122	54	0.0028	0.44		
	207	88	0.0045	0.42		

¹ K_c (first order) = $1/t \log a/a - x$, where a is the initial substrate concentration and x is the fraction hydrolyzed in time t .

² K_D (zero order) = x/t . Figure in parentheses was not used in computing average.

³ C_D [proteolytic coefficient (14)] = $K_D/\text{enzyme concentration}$.

mM per ml.) and that the entire course of the reaction, which is accurately measurable, proceeds in the first stage as a reaction of the zero order. This is in direct contrast to all other proteolytic enzymes whose reaction kinetics have been investigated, since in those cases the reaction proceeds in the second stage as a reaction of the first order even at the highest substrate concentrations that can be employed.

It should be possible to demonstrate the appearance of the second stage of the arachain-BAA reaction, provided means could be devised to make accurate measurements of the rate of hydrolysis at the extremely low substrate concentrations that would be required. The method of measurement employed in this work is not sufficiently accurate for this purpose at substrate concentrations significantly lower than that used in the experiments described. To demonstrate the appearance of the second reaction stage, another means of approach, namely, the use of a higher ratio of enzyme to substrate, might appear to be feasible. This approach has not been successful, because thus far it has not been possible to obtain arachain preparations sufficiently more potent than those used in the experiments reported to cause a significant increase in the enzyme : substrate ratio.

Inasmuch as K_D , expressed as per cent of substrate split per minute, is an accurate measure of the rate of hydrolysis of BAA by arachain, the establishment of a convenient enzyme unit for the assay of enzyme preparations can be accomplished quite readily. In this paper the results for enzymatic activity (Tables III, V, VI; Figs. 1, 2) have been

TABLE VI

Effectiveness of the Presence of Substrate in Retarding the Thermal Destruction of Arachain at 40°C.

Substrate, BAA; pH 6.0.

Enzyme preparation	Duration of hydrolysis min.	Extent of hydrolysis per cent	K_D
I	58	25	0.43
	120	52	0.43
	180	77	0.43
	240	95	0.40
II	77	13	0.17
	185	36	0.19
	300	57	0.19

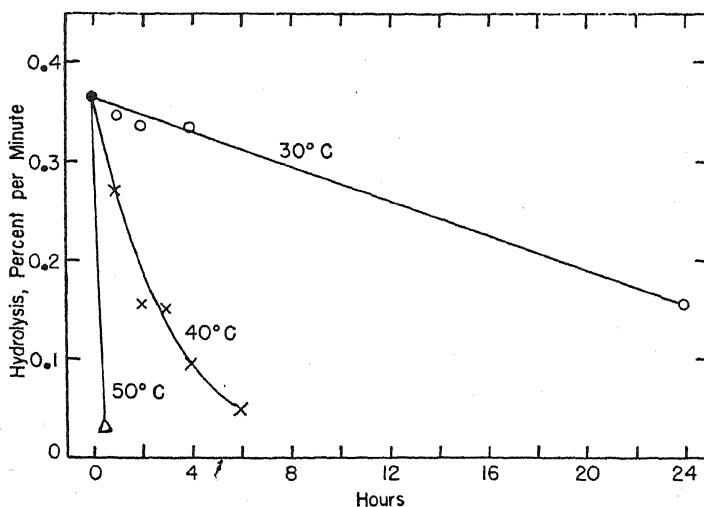


FIG. 2

Thermal Destruction of Arachain
pH 6.8 in 0.5 M sodium chloride; substrate, benzoyl-L-arginine amide.

expressed either in terms of K_D or in terms of an arachain unit, which was arbitrarily chosen to be equivalent to the amount of enzyme required to yield a K_D of 0.25 (or to hydrolyze 3×10^{-4} *mM* of substrate per minute) under the conditions of the test. It is noteworthy that, because the value of K_D remains constant even up to the point where 90 per cent of the substrate has been hydrolyzed, considerable latitude is afforded in the selection of the most suitable hydrolysis period for accurate assay.

Thermal Lability of Arachain

The stability of the enzyme at various temperatures was determined in the following manner. A freshly prepared sample of purified arachain (1.5 units per ml., 30°C.) in 0.5 *M* sodium chloride was divided into 3 portions, and one portion was allowed to stand at each of the following temperatures: 30°, 40°, and 50°C. One-ml. portions were removed at intervals for assay. As usual, BAA was used as the substrate at 30°C. and at pH 6.0. The results, shown graphically in Fig. 2, indicate that enzymatic activity disappears very rapidly when arachain is heated at 40°C., in the absence of substrate, and decreases at an appreciable rate, even at 30°C. However, this behavior does not constitute a serious handicap in testing the activity of the enzyme, since the presence of the substrate (BAA) retards thermal destruction of the enzyme sufficiently to permit accurate measurement of the rate of hydrolysis over relatively long periods, even at 40°C. The protective action of the substrate is evident from the results shown in Table VI; it may be noted that the value for K_D remains constant, even in dilute enzyme solutions and even after 4–6 hours at 40°C. when the substrate is present. In other words, arachain retains its activity in the presence of the substrate under conditions that would destroy 70–80 per cent of the enzyme in the absence of the substrate.

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SUMMARY

Peanut meal has been found to contain a proteolytic enzyme that rapidly hydrolyzes benzoyl-*L*-arginine amide to benzoyl-*L*-arginine and ammonia. The name arachain has been suggested for this enzyme.

Arachain is present in both peanut cotyledons and germs but to a greater extent in the former. It is absent from peanut skins. Germination for periods as long as 96 hours results in a significant, but not marked, increase in the amount of arachain present in the peanut.

Arachain preparations have been obtained that are approximately 20 times as potent as peanut meal and contain approximately 50 per cent of the original proteolytic activity of the meal.

Arachain is not activated or inhibited under conditions commonly used for the activation or inhibition of the papainases; it has optimum activity in the pH range 6.5–7.5, and is rapidly destroyed at 40°C.

The hydrolysis of benzoyl-*L*-arginine amide by arachain is a reaction of the zero order.

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Book Reviews

Colloid Chemistry. Vol. V, Theory and Methods, Biology and Medicine. Collected and edited by JEROME ALEXANDER. Reinhold Publishing Corporation, New York, N. Y., 1944. Seventy authors and 1256 pp. Price \$20.00.

Volume V, the latest addition to Jerome Alexander's series of publications on Colloid Chemistry, is of the same standard as the previous volumes. It is in two parts, I. Theory and Methods, II. Biology and Medicine. Although nothing is said to imply that the first part on theories and methods bears on biological and medical research as well as on theoretical and commercial chemistry, yet, with the possible exception of two or three chapters, the entire volume is directly applicable to biology and medicine.

There is a total of sixty chapters covering 1217 pages. Alexander starts the book with a chapter on Successive Levels of Material Structure. The chapter has a strong philosophical flavor. Its author cites Spencer's relativity of knowledge, Einstein's relativity of physical measurements, and the biologists' relativity of sense perceptions as illustrations of levels in the application of scientific principles. He then discusses the nature of so-called facts, the uncertainty of which is too little appreciated. With this the reviewer fully agrees, but not with some of Alexander's examples. Thus, he discusses "dowsing" or the search for water with the aid of diving rods. There is no scientific evidence that facts are involved in dowsing. For authority, Alexander cites J. J. Thomson. It was another physicist by the name of Thomson who believed that man cannot fly. The reviewer is in agreement with both Alexander, and Hamlet, Prince of Denmark, whom Alexander quotes, that there are more things in heaven and earth than are dreamed of, a truism which William Thomson did not and many like him still do not realize, but when taking a flight into the blue one must not soar too high. It is well in all philosophical interpretations of scientific hypotheses to have substantial data on which to base one's speculations.

The best of this first chapter by Alexander is a sentence near the end wherein it is said that much of the "mystery of life" arises from an inability—the reviewer is tempted to add here, and often an unwillingness—to descend to levels of structure where basic life processes can be coupled with chemical knowledge.

The second chapter is by W. D. Harkins on surface films. He states he was the first to present the idea of molecular orientation in surfaces and reproduces a page from his lectures of 1913 to prove it. Harkins does a favor to the biologist in considering films which are thicker than monolayers. The reviewer once asked Langmuir to do this because the protoplasmic membrane is a morphological structure often capable of being lifted off. If such a layer determines the selective permeability of cells, then something of the formation of interfaces which exceed molecular dimensions must be known. Harkins rejects as incomplete Rayleigh's theory that all liquids spread on the surface of clean water. He also rejects as in error Langmuir's theory that non-polar liquids do not spread on water whereas liquids with molecules

containing a polar group and a non-polar group do spread. Harkins then gives his own theory based on "duplex" spreading, which, he says meets all situations. The condition which determines whether or not an oil will spread is one depending on how well the oil "likes" itself; if the oil likes itself better than it does water, then it will not spread, whereas if it likes water better than itself, it will spread. Unfortunately, Harkins expresses the term "likes" as a thermodynamic quantity which is of no help to the biologist and of little help to the average physicist and chemist. The reviewer has often wondered if any situation is wholly clear in the mind of its author, still less so in other minds, if it can be expressed only as a mathematical quantity. Thus, when the biologist asks of the physical chemist, what is the "activity" of the hydrogen ion, he is told that it is *alpha*, and that is all that he is told.

The chapter by Harkins covers 87 pages; in it he presents methods of measuring film pressures; the viscosity of monolayers; the nature of monolayers in which the molecules lie flat and those in which the molecules are vertical; the deposition of monolayers to form multilayers; the function of the hydrogen bond; and the electric potential of monolayers and multilayers, the former are of the order of 60 mv.

A chapter by McBain is always a welcome contribution for the biologist, and it is likely to be on soaps, as is the third chapter in Alexander's Colloid Chemistry. The reviewer has heard the physical chemist express contempt for colloid chemistry, a subject which some chemists regard as not only composed of lawless systems but of lawless chemists as well. The reviewer also recalls quite another statement by that arch non-Newtonianist among us, W. D. Bancroft, who said that he alone among all electrochemists gave consideration to high concentrations of strong electrolytes, whereas others religiously kept to the dilute solutions because they alone obeyed the solution laws. Apparently McBain has had similar experiences for he says: "Thirty years ago it was assumed that colloids had no very definite properties. . . . It was supposed that a colloid could not conduct; nor could it have a definite quantitative, easily measurable effect on lowering the freezing point or vapor pressure." McBain then adds that the study of pure soaps has shown their solutions to have definite reproducible properties like those of a simple salt, and that colloidal electrolytes are stable in the strict thermodynamic sense. From this he concludes that soap solutions are colloidal electrolytes and that such a class of matter must be recognized. McBain continues with a discussion of the electrical and osmotic behavior of soaps, with examples of other colloidal electrolytes, and some factors of detergency, with applications.

The next several chapters are on Electron Diffraction, Analysis of Molecular Structures with the Aid of X-rays, Microradiography, and the now much written of Electron Microscope. All are excellently written, but with little mention of their possible biological bearing. That the electron microscope is of biological use the reviewer does not question, but these uses are as yet not very numerous, nor does the present volume make them convincing. The photographs of single molecules of proteins, tobacco mosaic virus, and normal serum are, like most electron microscope photographs, mere shadows. Somewhat better are the photographs of collagen fibrils.

The chapter on Rheological Properties of Colloidal Systems recalls to the writer the first meeting at the Bureau of Standards in Washington of the then newly formed Society of Rheology, where he advanced the hypothesis that all non-Newtonian fluids are elastic. For this he was severely criticized because the first non-Newtonian

liquids studied were Hardy's hydrocarbon oils, and a hydrocarbon oil cannot be elastic. Being a biologist the reviewer humbly returned to his chair subdued by so large and impressive an audience of physicists and chemists. Today we make rubber of hydrocarbons.

Eyring and Powell's discussion on the true and anomalous viscosity of liquids presents surface films and the theory of lubrication. Also included is that unfortunate word "plasticity" which the reviewer had hoped would be dropped in time. A word is needed to characterize fluids which require an initial stress to start flow, but plastic is not a suitable term, for plastic substances are those which can be deformed without exhibiting a tendency to return to the original shape, and this is precisely the contrary characteristic of non-Newtonian substances. Putty is plastic; gelatin, which exhibits anomalous flow, is not plastic. The reviewer's early contention that elastic solutions, such as those of some soaps, do not and cannot show true viscous flow, whereas non-elastic solutions are Newtonian, precludes use of the word plastic. Experiments bear this out.

If several chapters are passed over, as must be done in a brief review of so large a book, one comes to a chapter with a purely chemical subject which bears directly on biology; it is the Elasticity of Rubber. Unfortunately, though this subject could be of great help to biologists, it is handled in a manner which makes it incomprehensible to all except the specialist. When Freundlich or Hatschek wrote of gels, the biologist grasped with ease the concepts and understood the controversies, but when Guth tells us that a gel is an insoluble, infusible, three-dimensional polymer, biologists are none the wiser, even though they fully comprehend each of the words used, but why a gel is just that and nothing else is not made clear. However, some ideas may be gleaned by the biologist from even so highly technical a chapter: thus, Guth says, "in raw rubber we do not have a bundle of isolated long-chain molecules. Rather, the long-chain molecules form a coherent mass by some sort of cross-linking. There must exist strong forces between the molecules to prevent their slipping past one another. Otherwise raw rubber could not be elastic, but would show as much plasticity as beeswax and chewing gum, which substances, when stretched, do not retract to any extent." The reviewer is delighted to see the word plasticity correctly used and not in its new rheological sense. Cross-linking is one of the most constructive conceptions which chemists have given us in their treatment of the structure of elastic matter whether rubber, protein, or protoplasm.

The succeeding chapters on The Vitreous State, High Mechanical Stress, Sonic and Ultrasonic Waves, The Cyclotron, and The Betatron, all have their biological application though this is only lightly, if at all, touched upon.

The chapter on The Electrophoretic Study of Proteins by MacInnes and Longworth is an excellent and complete one dealing primarily with the instrument and method of the authors, but also describing other, particularly the Tiselius, techniques. The work on the purification of ovalbumin, the discovery of three globulins in egg white, the electrophoretic study of blood, and the isolation of pure hormones is of so great a significance to biology and medicine that some indication of it should be given even though it can at present be only vaguely predicted. Unfortunately the authors do not do so.

Only three of the remaining chapters which complete Part I of the volume have rather specific biological application. That on Selective Adsorption and Differential

Diffusion is one of these though no reference to its biological implications is made. The subsequent chapter on Liesegang Rings treats of a classical subject with some biological references.

The Fixing of Dates of Past Events by Tree Rings and Varves (varves are laminated deposits of glacial clays) is a presentation of the familiar tree-ring chronology of A. E. Douglass. Douglass was an astronomer interested in sun-spots; he found that the periodicity in sun-spots is reflected in tree growth. Such a subject is rather far afield in a volume on colloid chemistry.

The reviewer enters upon a discussion of Part II of Jerome Alexander's book with much trepidation. The application of Colloid Chemistry to biology and medicine has been the life work of the reviewer; this makes a summation of 690 pages on the subject at once both an easy and an extremely difficult task. To be confronted with chapters on Proteins by Astbury, Photosynthesis by Rothemund, Starch Chemistry by Hixon and Rundle, Muscle by Meyerhof, and thirty others of the same category, leaves a reviewer with a feeling of utter despair. No more than a light resumé of several of the more important chapters will be attempted.

It is fitting and proper that the first chapter should be by Professor W. T. Astbury on the X-ray analysis of proteins. If the reader is interested in the application of physical chemical research to biology, in particular, the structure of protoplasm, he should certainly familiarize himself with the work of Astbury. Astbury has more than the restricted interest of a physicist in his subject, he sees in it the biological aspects as well; furthermore he is ever ready to apply his research to biological problems. Astbury's viewpoint is the antithesis of the smug hopelessness with which some scientists view so basic a problem as the structure of living matter. The biological significance of Astbury's work is best put in one of his own sentences: "The precise nature of the α form and what happens during the reversibly elastic transformation of the β form are the source of the great interest and importance of the keratin-myosin group, for the first promises the key to the intramolecular folds of the corpuscular proteins, and the second is probably no less than a particular manifestation of the molecular mechanism involved in the contraction of muscle."

The second chapter of Part II is by Jerome Alexander on Catalysis as a Biological Factor. Alexander has long been interested in catalysis as a fundamental, if not *the* fundamental, vital reaction. Certainly, catalytic action has many properties, such as synthesis, which are ordinarily attributed only to living matter. Some such catalytic view of vital processes must be taken if life is to be analyzed on a strictly physical and chemical basis. Alexander presents a catalytic interpretation of cancer, and adds that the specifically effective abnormal catalysts responsible for cancer are hereditably reproduced. This, of course, is true only if cancer, whether produced by catalysts, gene mutations, or otherwise, is heritable, and this is not yet proved. There is, however, one contention by Alexander, involving a heritable quality with which the reviewer is in full agreement, namely, that there are cytoplasmic as distinct from chromosomal qualities which are inherited. Alexander says: "Catalyst modification . . . offers a simple but potent mechanism whereby non-genic as well as genic changes may be heritably transmitted."

If the biologist has not at hand one of Rothemund's chapters on photosynthesis here is his opportunity to possess it, and possess it he certainly should. The reviewer, who has long maintained that protoplasm is radioactive, and that the emanation of

such well known forms of energy as ultraviolet light from protoplasm is no more extraordinary than the emission of heat and light by organisms, cannot help but view with pleasure the frequent references in modern literature to the radioactivity of tissues. Thus, Rothemund quotes work wherein the radioactivity of the carboxyl group assumes a major role in photosynthesis.

No volume edited by Jerome Alexander could be devoid of poetry, beauty, and humor. To illustrate this delightful quality in Alexander's writings, I quote his reference to the statue of Bernini in the Borghese Gallery in Rome depicting "the stalwart Aeneas carrying the shrunken form of his aged father Anchises and leading his chubby son Ascanius," which, Alexander says, "beautifully illustrates the results of the complicated colloid chemical changes involved in aging." That there is poetry and beauty in this reference, I am sure the reader will admit. The humor lies in contemplating how innocent Bernini must have been of his contributions to gerontology, the colloid chemistry of aging.

All in all, Alexander's *Colloid Chemistry, Theoretical and Applied*, Vol. V, is a very interesting book, one certain to be of value to every research biologist and physician.

WILLIAM SEIFRIZ, Philadelphia, Pa.

Wood Chemistry. Edited by LOUIS E. WISE, Institute of Paper Chemistry, Appleton, Wisconsin; with fourteen contributing authors. Reinhold Publishing Corporation, New York, New York, 1944. viii and 900 pages. Price \$11.50.

This is an important book and a timely one, for there has not been a period in which interest in chemical utilization of wood has run as high. Since chemical utilization can and should be an important branch of general forest products utilization, it is well that this work, a compact compendium of basic chemical information, appears at the precise time when many major industrial factors contemplate wood as a chemical engineering raw material.

Yet, broad and deep as have been the changes since the appearance of the monograph by Hawley and Wise, "The Chemistry of Wood" in 1926 and Schorger's "Chemistry of Cellulose and Wood" in the same year, one can hardly escape the impression that we are still skirting the periphery of the pool of truth concerning wood.

Fundamentally, the past 20 years have seen some things rather well elucidated, others merely touched. The structure of alpha-cellulose, both physically and organically, may be considered as pretty well established; but the structures of the other celluloses and the hemicelluloses are still only dimly understood. As far as lignin is concerned, a multiplicity of formulae only strengthen the belief that lignin in the isolated form merely varies widely according to its method of isolation, and that there is as yet no substantial body of evidence as to the actual situation in the primordial wood fiber. Establishment of the existence of this or that number of various groups in the so-called isolated lignin molecule gives us only a fleeting glimpse of the structure of the primordial substance, assuming the existence of such structure within wood.

Nor can it be said that the anatomist, the physiologist or the cell-wall specialist have given any spectacular assistance in solving the riddle of the nature of wood.

Wood is all things to all people, apparently—a never-ending source of raw material both for industry and for argument.

The organization of such a work as "Wood Chemistry" with numerous contributors, all specialists, presents difficult problems to the editor. There must be compromises with space requirements in order to compress within one volume the relatively large masses of information covered. There must be balance among the various sections, and there should be avoidance of duplication and overlapping. As to balance, and coordination, only high praise can be given. There is considerable duplication and overlapping among the sections, which cannot be avoided. As a matter of fact, the treatment of various phases from the different points of view of the authorities is a distinctive feature of the kind of a work that is valuable.

Chapters 1 & 2. The Growth, Anatomy and the Physical Properties of Wood.

This is well done, but the reviewer feels rather strongly that this section has no place in a volume treating of the chemistry of wood.

Chapter 3. Cellulose, the Principal Component of the Cell Wall; Chemical Evidence Regarding Its Constitution—LOUIS E. WISE.

This is a highly compressed but adequate, scholarly and concise treatment giving the main elements of structural argument and pointing out unsolved problems. (67 references.)

Chapter 4. Plant Cellulose—A. G. NORMAN.

Dr. Norman cuts away most of the voluminous hocus-pocus that has cluttered up the literature and produces a lucid, well-written essay to the effect that (1) plant cellulose is an intricate affair, the composition of which varies according to the methods used in fractionation; (2) "celluloses" is a more nearly accurate term. (15 references.)

Chapter 5. Cellulose: Physical Evidence Regarding Its Constitution—H. MARK.

Professor Mark, eminently qualified for the task, has organized the voluminous literature on the subject in a thoroughly workmanlike manner. A reasonably good working knowledge of the complex subject can be obtained from this chapter at a saving of infinite labor. The narration of the derivation of our knowledge as to the nature of the cellulose particle and its fitting into general theory is fascinatingly told. (178 references.)

Chapter 6. Chemically Modified Cellulose—LOUIS E. WISE.

This chapter contains considerable duplication of material treated in other chapters. (43 references.)

Chapter 7. Cellulose Solvents and the Properties of Cellulose in Solution—A. J. STAMM.

This is a good development of this many-sided question from the colloid chemist's point of view. While one may quarrel with the adequacy of the point of view, the evidence and its interpretation is here admirably presented and well interpreted. (162 references.)

Chapter 8. Cellulose Compounds and Derivatives—EDWIN C. JAHN.

This chapter begins the development of making things from wood. It is not an industrial handbook but is an excellent fundamental treatment of industrial utilization with treatment of important industrial procedures adequate for a book of this kind. (235 references.)

Chapter 9. Hemicelluloses of Wood—A. G. NORMAN.

A lucid discussion of a very complex subject, properly dwelling on the more recent work that has gone so far to clear up the general nature of hemicelluloses. Includes a brief discussion of industrial possibilities. (46 references.)

Chapter 10. The Chemistry of Lignin—MAX PHILLIPS.

Dr. Phillips gives a good resume of reactions and work on the constitution of the various forms of lignin. He sums it up thus: "There has been considerable difference of opinion among the various investigators of the chemistry of lignin as to whether this substance belongs to the aliphatic, aromatic, hydroaromatic or heterocyclic series." This gives a sufficiently good impression of the chaotic condition of lignin chemistry. "It cannot be emphasized too strongly that it is entirely premature to propose any constitutional formula for lignin, considering our incomplete knowledge of the chemistry of this substance." (402 references.)

Chapter 11. The Chemistry of Cell Walls of Wood—WILLIAM M. HARLOW.

A brief chapter and adequate for inclusion. (18 references.)

Chapter 12. The Extraneous Components of Wood—ERVIN F. KURTH.

This chapter tries to compress within 60 pages a great deal of the content of Wehmer, Wiesner and similar works. The field is too broad and the material too widespread in the plant world to permit adequacy to be achieved in a treatment of this magnitude. Kurth makes numerous statements in that part of the chapter dealing with oils and resins that cannot be substantiated. In his treatment of pigments, tannins, sugars, etc., he is much stronger and apparently on more certain ground. (307 references.)

Chapter 13. Surface Properties of Cellulosic Materials—A. J. STAMM.

This is a treatment of the all-important wood-water relationship by a supreme authority in the field. Further, Stamm takes up swelling in non-aqueous materials, the basis for certain new forms of impregnation. His treatment of the drying of wood indicates the complexity of the subject with lucid explanation. It is significant that most of Stamm's bibliography has appeared since 1926. (252 references.)

Chapter 14. Introduction to Wood Analysis—RICHARD D. FREEMAN.

A treatment of various schemes for various purposes with the caution that they are tools only. (22 references.)

Chapter 15. Preparation of Wood Samples; Miscellaneous Determinations—RICHARD D. FREEMAN.

Treats of subdivision, water, ash and fractions. (118 references.)

Chapter 16. Determination of Cellulose—RICHARD D. FREEMAN. (88 references.)

Chapter 17. Total Carbohydrate Fraction of Extracted Wood. Pentosans and Hexosans in Wood—RICHARD D. FREEMAN. (110 references.)

Chapter 18. Determination of Lignin—MAX PHILLIPS.

In this very practical chapter, Dr. Phillips concludes that the best we can do is make approximate determinations only because we do not know what we are trying to determine. (60 references.)

Chapter 19. Analytical Data and Their Significance—RICHARD D. FREEMAN.

This chapter treats of the comparative value of analytical data, especially in the field of process control. There are valuable interpretations of the significance of analytical methods and findings to several fields of technology, especially pulping. (42 references.)

Chapter 20. Combustion of Wood—L. F. HAWLEY.

Dr. Hawley gives a short and concise treatment of this largest single worldwide use of wood. It is amazing that so little work can be reported in this field. (4 references.)

Chapter 21. Thermal Decomposition of Wood—L. F. HAWLEY.

There has been little work of importance between 1925 and 1943 which reflects the general attitude that the industry was dying. There is hope of its revival as a part of integrated utilization. (29 references.)

Chapter 22. Delignification of Wood—W. O. HISEY.

This is a theoretical treatment combined with considerable practical discussion of the general problems of pulping, bleaching, and pulp purification. It is not a technological handbook but an excellent resume of important basic information. (220 references.)

Chapter 23. The Chemical Behavior of Wood—EDWIN C. JAHN.

This chapter overlaps other chapters in some degree but contains much new material on the action of acids, salts, bases, oxidizing and reducing agents on wood. Both general fields—the industrial chemistry of wood and the use of wood as a structural material in chemical industry—are contained within the chapter. (245 references.)

Chapter 24. Decomposition of Cellulose and Hemicelluloses by Microorganisms—SELMAN A. WAKSMAN.

This extraordinarily complex subject is given a thoroughly scholarly treatment. Economic aspects of cellulose decomposition, however, are not very fully treated. The possibilities of industrialization based upon the use of microorganisms of wood are probably of sufficient importance to warrant fuller treatment. (145 references.)

Chapter 25. Decomposition of Lignin—SELMAN A. WAKSMAN.

This chapter relates lignin to humus without too much expansion of the obvious possibility for theoretical discussion. (48 references.)

J. A. HALL, Washington, D. C.

The Effect of Cations on the Activity of Alfalfa Pectinesterase (Pectase)¹

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INTRODUCTION

For the most part, those who have studied the enzymic de-esterification of pectin have regarded polyvalent cations (particularly calcium) as agents that participate in the process of gelation but not in the enzymic reaction proper—that is, de-esterification (1). Kopaczewski (3), however, stated in 1925 that in a neutralized mixture of purified pectin and dialyzed enzyme prepared from alfalfa, de-esterification occurred only in the presence of alkali or alkaline earth salts. Apparently his observation has been overlooked, possibly because his paper is concerned with several aspects of pectin coagulation, in addition to this qualitative observation. Indeed, his statement came to our attention only after we had rediscovered that electrolytes increase the activity of pectinesterase as much as 30-fold near pH 6 (4).

Evidently pectinesterase must be added to the large list of hydrolytic enzymes whose activities are increased by electrolytes. With some enzymes the effect is limited to specific ions. For example, arginase is activated by Mn, Co, Ni, and Fe under certain conditions

¹ The enzyme that acts on pectin and causes gelation in the presence of calcium has been known as pectase. Kertesz (1) proposed the name pectin-methoxylase for this enzyme, presumably because it is customary to speak of the methoxyl content of pectin. However, since the enzyme hydrolyzes the ester bonds in pectin we propose the name *pectinesterase*, which indicates the esterase character of the enzyme and avoids implication that the enzyme hydrolyzes glycosidic or other non-ester methoxy bonds. The unhyphenated term is used, following Glick and Glaubach (2), who used the terms *cholinesterase* and *atropinesterase* to designate the esterases acting on choline and atropine.

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but not by certain other ions (5), yeast phosphatase by Mg, Mn, Fe, Co, and Ni (6), pyrophosphatases by Mg but not by Mn (7), and salivary and pancreatic amylases by halides and nitrate but not by sulfate or phosphate (8, 9, 10). Activation of other enzymes has not been shown to be limited to a specific or a few specific ions, *e.g.*, the activation of cholinesterase by cations (11, 12) and of hyaluronidase (mucinase) by anions (13, 14). Except for those cases where electrolytes cause only minor differences in the rate of action and where they may cause peptization of the enzyme, the most attractive explanation of the activating effect of ions is that they are integral constituents of the enzyme. The effect of Fe, Co, Ni, and Mn on arginase activity (5), of Mg and Mn on leucyl peptidase activity (15, 16, 17), of Mg on phosphomonoesterase activity (18), and of several ions on amylase activity (9, 10) may be explained in this way.

The purpose of this paper is to describe some of the characteristics of the effect of electrolytes on pectinesterase activity and to discuss the mode of action of electrolytes, particularly cations, on the pectinesterase-pectin system. In contrast with the above-mentioned theory of ion activation, the results to be reported support the view that cations are not necessarily integral constituents of pectinesterase, but act by preventing or reversing the inhibition of the anionic pectin carboxyl groups, which in reality are reaction products. Thus cations appear to prevent the formation or to promote the dissociation, of inactive enzyme-carboxyl complexes. That "activation" by electrolytes may be a reversal of inhibition by ionic reaction products is suggested by the observations that the inhibition of enzymes by basic dye ions can be reversed by phosphate ion (19), that the inhibition of trypsin by soap can be reversed by calcium (20), that CaCl_2 decreases the association of toluidine blue and heparin (21), and, in general, that the formation of compounds that depend to a considerable extent on ionic forces is affected markedly by electrolytes.

MATERIALS AND METHODS

Substrate. Purified pectin was prepared by the method of Olsen, *et al.* (22) from 200 jelly grade citrus pectin. The ash content was reduced from 2.47 to 0.44%. On an ash-free and moisture-free basis, the CH_3O content was found by saponification (22) to be about 11%.

A 1% solution by weight of the purified pectin served as a stock substrate solution. In some cases unpurified 200 jelly grade citrus pectin was used. The aqueous solu-

ion, which had a pH of 2.9, was not neutralized until it was to be used because of the greater stability of the ester at the low pH. A decrease in CH_3O content of less than 0.2% occurred in two months.

Alfalfa Pectinesterase. The enzyme used in part of this work was a dry preparation made from alfalfa press juice by cold alcohol precipitation (cf. Mehltz, 23, 24). The solid was extracted with water or dilute NaCl solution and the residue discarded. More nearly complete extraction of the activity was obtained with dilute NaCl solutions. An equally satisfactory enzyme preparation was made by extracting one part of dried alfalfa (dried at $45^\circ\text{C}.$) with 7 parts of 0.1 *M* sodium acetate solution. The extract, separated from the residue by squeezing through cheesecloth, was centrifuged, filtered, and dialyzed. The clear filtrate of the turbid dialyzate possessed about $\frac{3}{4}$ and the precipitate $\frac{1}{4}$ of the activity.

Pectinesterase Activity Determinations. Some of the results reported in this paper were obtained by the method of Kertesz (25), modified to the extent that the electrolyte concentration was controlled and the reaction mixture was maintained nearly continuously at a pH that just gave a yellow color with methyl red (about pH 6.2) instead of being titrated to this point each 5 minutes.⁴ The pectin concentration in the reaction mixture was 0.5%, the volume was generally 20 ml., and the alkali used for titration was either $N/10$ or $N/50$ NaOH. The hydrolysis was carried out conveniently at room temperature since it was found that the enzyme activity varied only about 3% per degree at this temperature ($25^\circ \pm 1^\circ\text{C}.$). Whereas the average pH by the indicator method was about 6.2, it was estimated that the pH varied from about 5.5 to 6.8 during the course of the reaction due to non-uniform manipulation.

The method used in experiments carried out at pH values other than 6, and in those that required precise control of pH, was similar to the continuous titration procedure used by Alles and Hawes (26) for the determination of cholinesterase activity. The assembly of the glass electrode (Beckman), stirrer, burette, tube for conducting CO_2 -free air over the reaction mixture, and beaker was such that it could be lowered into a constant-temperature bath. Forty-ml. volumes of reaction mixture held in 100-ml. beakers were used ordinarily. The temperature of the bath was $30^\circ\text{C}.$ Errors due to the absorption of CO_2 at higher pH values were avoided by a gentle flow of CO_2 -free air over the surface of the liquid. At low electrolyte concentrations it was necessary to standardize the stirring and make corrections for a "stirring potential" in order to obtain the true pH of the solution. The correction was estimated from readings taken while the solution was stirred and while it was at rest. In a 0.5% pectin solution, the corrections applied to the observed pH reading varied slightly but were generally as follows: +0.2, +0.08, and +0.02 at ionic strengths of 0.005, 0.05, and 0.15, respectively. It was later found that the error was negligible when a sleeve-type rather than a wick-type calomel electrode was used. Suitable corrections in the titration values were made for chemical de-esterification, which

⁴ In the absence of appropriate amounts of electrolyte, pectin and enzyme samples having different electrolyte contents will give incorrect relative activities at this pH. The esterase activity of tomato juice (which contains considerable electrolyte), determined by the Kertesz method with unpurified pectin, is increased about 3-fold by making the reaction mixture 0.15 *M* with respect to Na ion.

occurs to an appreciable extent above pH 7, and for incomplete titration of liberated carboxyl groups below pH 5.5.

Pectinesterase Activity Units. One pectinesterase unit, [PE.u.], is defined as the quantity of enzyme that, at 30°C. and optimum pH, will catalyze the hydrolysis of pectin at an initial rate of 1 milliequivalent of ester bonds per minute in a standard substrate (0.5% citrus pectin containing 8–11% CH_3O) and 0.15 *M* NaCl. In the case of alfalfa enzyme the pH optimum in 0.15 *M* NaCl solution is near 6.0 and a pH between 5.6–6.4 should be used. Potassium oxalate may be added to 0.01 *M* if the calcium in the enzyme preparation interferes with the titrations by causing gel formation. The expressions [PE.u.]_{mg.}, [PE.u.]_{ml.}, [PE.u.]_{mg.P.N.} refer to the number of units per mg., per ml., and per mg. of protein nitrogen of the enzyme. The "E" is used in the symbol to emphasize esterase as distinguished from polygalacturonase (a glycosidase). The unit used by Kertesz, the amount of enzyme liberating one mg. of CH_3O per 30 minutes at 30°, is 930 times this unit.

Where possible ⁵ a unit based on milliequivalents of bonds acted upon by the enzyme per minute (cf. Northrop and Kunitz, 27) is favored by the authors for the following reasons: (a) It is a comparative measure of the catalytic ability of enzymes whether they are esterases, peptidases, or some other type of enzyme; for example, the activity of the dry alfalfa pectinesterase preparation previously mentioned had a specific activity, [PE.u.]_{mg.P.N.}, of 0.2, which is similar in magnitude to the activities of proteolytic enzymes (28).⁶ (b) The units per millimole of enzyme is numerically equal to the "turnover" number (29). (c) The units per mg. or ml. of enzyme material is convenient for the experimentalist who can readily and uniformly estimate the amount of chemical change to be expected from a given quantity of any enzyme standardized in this unit; for example, if a reaction requires 1 ml. of titrating solution per minute, the units in the reaction mixture aliquot are equal to the normality of the solution; thus, if a rate of about 1 ml. per 10 minutes of 0.1 *N* solution is desired he would use an amount of enzyme material containing $(1/10) \times 0.1$, or 0.01 unit.

⁵ Exceptions would include cases where the method of assay involves a change in physical properties such as viscosity, the stoichiometric relation to bonds altered being unknown. In cases where it is applicable the first-order reaction constant should, of course, be used.

⁶ This does not mean that the pectinesterase preparation is nearly pure but merely that on a gram weight basis this preparation is about as efficient as the purified proteinases.

RESULTS

Validity of Assay Method under Various Conditions. The results plotted in Fig. 1 show that the rate of de-esterification of pectin in 0.1 *M* NaCl solution by pectinesterases from two sources, alfalfa and pea vines, is practically constant at the methyl red end point, until the saponifiable CH_3O content is reduced to 5%. Unreported experiments revealed a linear relationship also for determinations made at pH values between 4 and 9 and at various NaCl concentrations. In these cases, however, the reaction was not followed beyond a 10% decrease in CH_3O content. Because pectic acid inhibits the activity

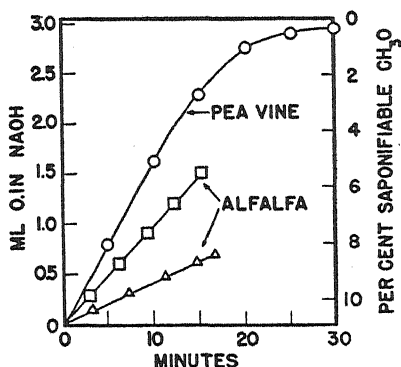


FIG. 1

Illustrative Time Curves for the De-esterification of Pectin by Alfalfa and Pea-Vine Pectinesterase in the Presence of 0.1 *M* NaCl at the Methyl Red End Point

of this enzyme at low electrolyte concentrations, it seems likely that the linear relation would not hold over as great a range of de-esterification at low electrolyte concentrations as at higher concentrations.

The initial rate of reaction was found to be linear with enzyme concentration over a 5-fold concentration range (0.05 *M* NaCl). At either 0.15 *M* NaCl or at 0.005 *M* Na ion (due to neutralization), the activity for two ml. of dialyzed enzyme solution was twice that for one ml., within experimental error. Also the activity per ml. at 0.002 *M* CaCl_2 remained constant over a 4-fold range of enzyme concentration. The slopes of the alkali *vs.* time curves can be used, therefore, in investigations of the influence of various factors on the enzyme activity.

The Electrolyte Effect is Due to Cations. Fig. 2 illustrates the influence of eleven salts on the activity of a dialyzed water-clear alfalfa-enzyme solution at approximately pH 6 (methyl red). The curves are somewhat inaccurate at the lower salt concentrations because here the activities change markedly with pH (see section on activity-pH rela-

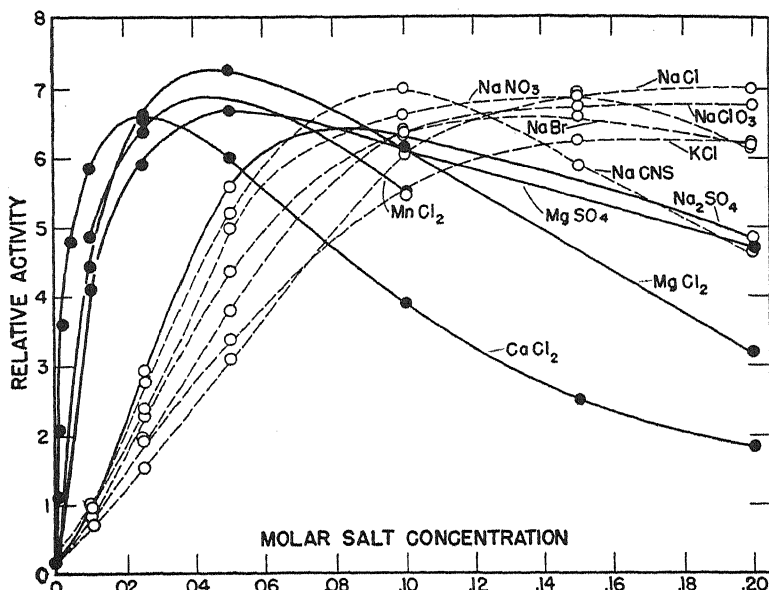


FIG. 2

The Effect of Salt on the Activity of Dialyzed Alfalfa Pectinesterase Acting on Purified 0.5% Pectin at the Methyl Red End Point

The dotted lines represent monovalent inorganic salts. The solid lines represent divalent salts.

tion). The salts are practically interchangeable at optimum salt concentrations, since nearly the same maximum activity was obtained for all of the salts. The relative activity values ranged from 6.2 to 7.2. The maximum activities for LiCl and BaCl₂, although not shown on the figure, also fall within this range. The increased activity is due chiefly to the cations since, for example, CaCl₂ and NaCl at equal (low) chloride concentrations cause very different increases in activity. A detailed study of the inhibition that occurs at high concentrations of divalent cations was not made.

The data in Table I show the relative effectiveness of the various salts in increasing the enzyme activity at pH 5.7 from about 3 to 14% of the maximum activity; that is, in the salt-concentration region where the activity increases linearly with salt concentration. The data confirm the results reported in Fig. 2 in that the monovalent (dotted lines) and divalent cations fall into two classes, the divalent cations being much more effective in increasing the activity at this pH. The

TABLE I

The Concentrations and Ionic Strengths of Various Electrolytes that Increase Pectinesterase Activity from About 3 to 14% of Maximum (pH 5.7 \pm 0.1)

Electrolyte*	Concentration $M \times 10^2$	Ionic Strength $\mu \times 10^2$
LiCl	2.0	2.0
NaCl	1.3	1.3
Na ₂ SO ₄	0.65	2.0
KCl	1.4	1.4
NH ₄ Cl	1.5	1.5
MgCl ₂	0.10	0.3
MnCl ₂	0.08	0.24
CaCl ₂	0.07	0.21
BaCl ₂	0.08	0.24

* FeCl₃ and Th(NO₃)₄ were observed qualitatively to give some activation but precipitation of the pectin was so great even at low concentrations that quantitative figures were not obtained.

results with FeCl₃ and Th(NO₃)₄ were unsatisfactory because of formation of precipitate. It is evident from Column 3 in Table I that the effect is not simply one of ionic strength.

In the studies reported below only the Na cation was used. If divalent salts were used the results would be expected to differ quantitatively but not qualitatively.

The Cation Effect is Independent of Enzyme and Pectin Concentration. It was noted in the section on validity of the assay method that the usual direct relationship between activity and enzyme concentration was found at several electrolyte concentrations. Therefore, the percentage increase in activity caused by cations must be independent of the enzyme concentration.

Table II shows that varying the substrate concentration at pH 5.7 does not affect the relation between cation concentration and activity.

In carrying out the experiments at low cation concentrations, the Na ion concentration was maintained constant by taking account of the amount of Na ion added in neutralizing the pectin. The relation between activity and substrate concentration at both 0.025 *M* and 0.20 *M* Na ion could be represented by the Michaelis-Menten equation:

$$v = \frac{V_{\max.}[\text{pectin}]}{K_m + [\text{pectin}]}$$

The value of $V_{\max.}$, the maximum velocity at high pectin concentrations, at the higher Na concentration was about 3.5 times that at the lower concentration, but the values of the dissociation constant,

TABLE II

The Effect of NaCl at Various Pectin Concentrations on Pectinesterase Activity at pH 5.7

Pectin concentration per cent	Activity at 0.025 <i>M</i> Na ⁺		Activity at 0.20 <i>M</i> Na ⁺		Ratio of observed activities*
	Observed	Calculated $K_m = 0.04$ $V_{\max.} = 2.65$	Observed	Calculated $K_m = 0.04$ $V_{\max.} = 9.4$	
0.5	2.5	2.4	8.8	8.7	3.52
0.15	2.0	2.1			
0.09	1.8	1.8	6.3	6.5	3.50
0.05	1.5	1.5	5.3	5.2	3.53
0.035	1.25	1.24	4.6	4.4	3.68
0.02	0.90	0.88			

* The ratio of $V_{\max.}$ at the two salt concentrations is 3.55.

K_m of the postulated enzyme-substrate complex, were about the same, 0.04%. The small variation in the ratios of the observed activities shown in the last column of Table II reflects this similarity in K_m values. In spite of the good agreement, the authors feel that the data are not critical enough to rely on the K_m values being identical within about 25%. This uncertainty arises from the fact that the activity at pH 5.7 and low electrolyte concentrations is very sensitive to pH, as shown in the following section. However, it is evident that cations do not alter markedly the equilibrium step ($E + S \rightleftharpoons ES$) postulated generally for enzyme reactions.

Cations Affect the Relation between pH and Enzyme Activity. With increasing concentrations of NaCl the activity-pH curves are shifted to lower pH regions (Fig. 3). As the pH is increased, maximum activi-

ties are attained at about pH 5.6, 6.0, 7.5, and 8.0 in the presence of 0.15, 0.10, 0.04, and 0.02 *M* Na ion, respectively. However, the maximum activities are all the same, within experimental error. On the acid side of the optimum, the activity-pH curves resemble the curves for most enzymes. The optima are broad, extending over more than 2 pH units. Activity determinations were not made above pH 9 be-

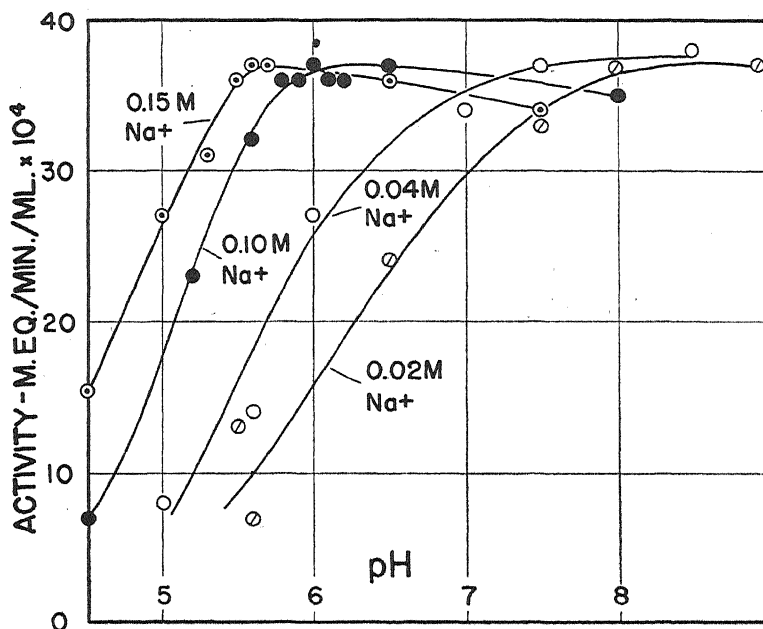


FIG. 3

Activity-pH Curves for Alfalfa Pectinesterase at 0.02, 0.04, 0.10, and 0.15 *M* Na Ion. These concentrations were realized from the NaOH used to neutralize the pectin plus the requisite amount of NaCl

cause of the large amount of chemical de-esterification that occurs in the more alkaline solutions. The activity values have been corrected for alkaline de-esterification above pH 7 and for incompleteness of titration below pH 5.5.

It is evident from Fig. 3 that the increase in activity caused by adding a certain amount of NaCl to a reaction mixture depends on the pH of the mixture. In fact, the addition of an amount of NaCl that

resulted in an increase in activity at, for example, pH 6, caused some inhibition at pH 8. Thus the results reported in Fig. 2 would have differed quantitatively had a different pH been used. A similar condition exists for mucinase (14), amylase (9), and other enzymes whose pH optima are shifted by the addition of salt or buffer. However, in contrast with the pectinesterase results, the activities of the other enzymes at the pH optima corresponding to different salts or salt concentrations were generally different.

TABLE III

Effect of NaCl on the Inhibition of Pectinesterase by Sodium Pectate

Na ion conc. <i>M</i>	Pectate conc. ^a	Relative activity	Inhibition by pectate
	Activity determined at pH 5.7		
	<i>per cent</i>		<i>per cent</i>
0.015	{ 0.0	(11) ^b	—
	{ 0.23	5	55
0.025	{ 0.0	31	—
	{ 0.45	4	87
0.04	{ 0.0	42	—
	{ 0.23	17	60
	{ 0.45	9	79
0.15	{ 0.0	100	—
	{ 0.23	86	14
	{ 0.45	73	27
0.20	{ 0.0	100	—
	{ 0.23	83	17
	Activity determined at pH 8.5		
0.015	{ 0.0	100	—
	{ 0.23	91	9

^a Calculated as pectic acid.

^b Obtained by interpolation between 0.005 and 0.025 *M* Na ion.

Cations Counteract Inhibition Caused by Sodium Pectate. Sodium pectate was found to inhibit markedly the action of pectinesterase only under suboptimal conditions of salt or pH. Table III shows that 0.23–0.45% pectate at pH 5.7 and low NaCl concentrations caused from 55 to 87% inhibition, whereas at higher salt concentrations or at a high pH (8.5), the inhibition was much less (about 15%). Thus

the same factor that is affected by cations in the absence of pectate appears to be involved in the inhibition of pectinesterase by pectate. The pectin substrate, which contains some free carboxyl groups, of course, might be thought of as consisting of pectic acid and completely esterified pectic acid.

Elution of Enzyme Adsorbed on Celite Simulates the Counteraction of Sodium Pectate Inhibition by NaCl. The enzyme was adsorbed readily on negatively charged diatomaceous earth in the absence of salt. However, adsorption was largely prevented in the presence of salt. Table IV shows that nearly 90% of the enzyme in a dialyzed solution

TABLE IV

The Effect of NaCl on the Adsorption and Elution of Pectinesterase on Celite (Analytical) at pH 4.75

10 ml. of the clear filtrate (paper) of dialyzed enzyme (E) solution plus 2.5 ml. of H₂O or 1 M NaCl were passed twice through 200 mg. of Celite (dimensions 1/3 × 1.5 cm.)

Solution and treatment	Activity of solution [PE.u.] _{ml.} × 10 ⁴	Activity retained on celite ^a per cent	Color of solution
1. E + H ₂ O or NaCl, no treatment	3.4	—	Yellow
2. E + NaCl, passed through Celite	3.0	12	Yellow
3. E + H ₂ O, passed through Celite	0.44	87	Yellow
4. Eluate obtained by passing 12.5 ml. of 0.2 M NaCl through Celite from No. 3	2.0	28	Colorless

^a Calculated by difference.

at pH 4.75 was adsorbed on a small amount of Celite analytical filter aid, and that at least 2/3 of the adsorbed enzyme was eluted by washing the Celite with a dilute salt solution. It is of interest that a purification of the alfalfa extract from the yellow flavone color, which is not adsorbed, was thus obtained. This enzyme, as are many others, appears to be susceptible to purification by relatively simple adsorption techniques.

It has been reported previously that the esterase is adsorbed on decolorizing charcoal. Kertesz (1) found that the esterase from tobacco was adsorbed almost quantitatively, but Mehltz (24) reported that the esterase from alfalfa was not adsorbed. Although these differences might have been due to differences in the enzyme it is more likely that they were due to differences in experimental conditions, e.g., salt concentration.

DISCUSSION OF THE MODE OF ACTION OF CATIONS ON PECTINESTERASE

The results do not show that cations are essential for the realization of full activity of pectinesterase but show rather that full activity can be obtained at a lower pH in their presence. A mechanism to explain how cations effect this shift in activity-pH relation of pectinesterase will be discussed. First, it may be noted that the increase in activity caused by salt at low pH or by raising the pH at low salt concentrations is not a peptization of the enzyme, since crystal-clear solutions of the enzyme were used and since the addition of salt did not alter the slight Tyndall effect given by the solution.

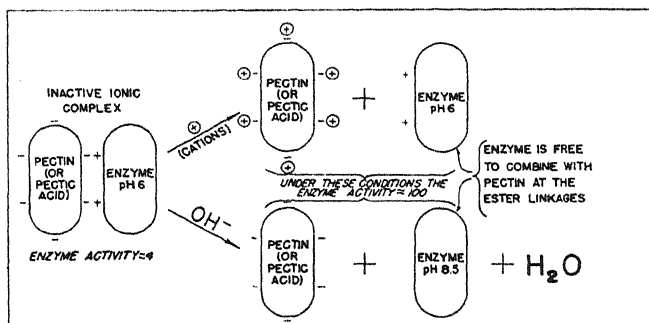


FIG. 4

Diagrammatic Representation of a Mechanism of the Effect of Cations and pH on Pectinesterase Activity

The similarity in the NaCl and pH-activity relations of pectinesterase inhibited by pectic acid and of pectinesterase in the presence of substrate only, suggests that the cations function by preventing inhibition. Removal of inhibition rather than specific activation is also indicated by the fact that cations near pH 6 increase the activity to about the same value realized near pH 8.5 in the absence of cations, other than those due to neutralization. A mechanism that is consistent with the results is shown diagrammatically in Fig. 4. The cations are represented as freeing the enzyme from the inactive ionic (pectin-carboxyl) complex, as if by competitive ion exchange. This mechanism also explains the adsorption of the enzyme on Celite and its elution by NaCl. The formation of a pectin-cation complex such as that in

Fig. 4 is supported not only by our knowledge of the effect of electrolytes on colloids but also by the finding that cations accelerate the rate of alkaline de-esterification of pectin several-fold, apparently by decreasing the electrostatic repulsion of pectin for anions so that more OH ions collide with the pectin (30). In the diagram the hydroxyl ions are represented as freeing the enzyme from the inactive complex by changing the charge on the enzyme. Although the form of the enzyme at pH 8.5 is represented as uncharged (*i.e.*, isoelectric) and at pH 6 as positively charged, it is intended only to signify the fact, which must be the case, that the cationic strength of the enzyme decreases as the pH is increased—that is, it has less tendency to combine with anions. It is not implied, nor is it necessary for the hypothesis, that the isoelectric point of the enzyme be at pH 8.5, or even above pH 7.

An essential and perhaps unusual feature of this hypothesis is that different ionic forms of the enzyme (*e.g.*, those at pH 6 and pH 8.5) exhibit about the same activity. However, if it is assumed that the reaction between the substrate and the enzyme is not an ionic reaction, it then might be expected that different ionic forms would have about the same activity, so long as conditions are such that the forms do not react ionically with substances in the reaction mixture to give inactive complexes.

The failure of anions to reverse inhibition by competing with pectin or pectic acid for the enzyme (*cf.* Fig. 4) may be attributed to the strong anionic character of the polyvalent pectin and pectic acid compared with the inorganic anions. On the other hand, the cationic enzyme, which contains some negative as well as positive charges, is probably not the strong ion that pectin is, and therefore the inorganic cations may be able to compete with the enzyme and thus dissociate the inactive ionic complex.

As is generally true, more than one mechanism may be consistent with a given set of kinetic results. In this case it might be assumed that cations and OH ions "activate" by causing the rupture of zwitter ions in the enzyme and that the inhibiting effect of the anionic pectin and pectic acid diagramed in Fig. 4 represents only a part of the mechanism of action of cations on pectinesterase. Additional studies will be necessary to resolve the possible mechanisms. When time is available it is planned to make further studies with purified enzyme preparations in order to eliminate any effects of impurities and to make possible chemical and physical studies on the enzyme protein.

SUMMARY

The activity of pectinesterase at pH 5.7 is about 30 times as great in the presence of 0.2 *M* monovalent cations or 0.02 *M* divalent cations as in the absence of cations, but at pH 8.5 these concentrations of cations are practically without effect on the activity. The enzyme is only slightly inhibited by sodium pectate at pH 8.5, yet it is markedly inhibited at pH 5.7 in the absence but not in the presence of cations. The enzyme-substrate dissociation constant at pH 5.7 is about the same (0.04%) at 0.025 and 0.2 *M* Na ion. The enzyme is adsorbed on Celite near pH 5 and is eluted in dilute salt solution.

A hypothesis correlating these observations is discussed. It is based on the assumptions, (a) that cations prevent inhibition by the pectin carboxyl groups by forming cation-carboxyl complexes, and (b) that the form of the enzyme existing in alkaline solutions has a decreased tendency to form an inactive complex with the carboxyl groups.

We are indebted to L. R. MacDonnell, Kay Denman, and Margery Doerr for assistance in the latter part of the work.

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Intestinal Synthesis of B Vitamins by the Rat *

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INTRODUCTION

A renewed interest has developed during the past few years in the factors which influence the elaboration of certain B vitamins by intestinal microorganisms and the importance of these factors to the host. Several techniques have been used to approach this problem: intestinal flora studies, Abdel-Salaam and Leong (1), Crecelius and Rettger (2), and Gant, *et al.* (3); cecectomy experiments, Griffith (4), Taylor, *et al.* (5), and Day, *et al.* (6); sulfa drug feeding work, Black, *et al.* (7), and Welch and Wright (8); and dietary modifications, Guerrant, *et al.* (9), Morgan, *et al.* (10), and Mannering, *et al.* (11).

In an attempt to correlate some of these techniques and to extend studies on conditions affecting intestinal synthesis, the following experiments were carried out: the effect of cecectomy on the growth of young rats fed synthetic, high sucrose diets which contain limiting amounts of the B vitamins, the effect of cecectomy and sulfasuxidine feeding together with modifications in the kind of carbohydrate on the total thiamine and riboflavin excretions, and the effect of subminimal vitamin levels on growth and thiamine and riboflavin excretion.

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EXPERIMENTAL

To study the effect of cecectomy, eighty male weanling rats were obtained from Sprague-Dawley. Thirty-six of the animals were cecectomized and the normal and cecectomized rats were divided into nine groups of four rats each. The basal ration used in this experiment was composed of sucrose 73 per cent, purified casein 18 per cent, corn oil 5 per cent, salts IV 4 per cent; and thiamine 250 $\mu\text{g.}$, riboflavin 300 $\mu\text{g.}$, calcium pantothenate 2 mg., nicotinic acid 250 $\mu\text{g.}$, pyridoxin 250 $\mu\text{g.}$, and choline 10 mg. per 100 g. of ration. Deviations or supplements to this ration are described in Table I. The average growth per week for each group over a period of 6 weeks is also shown in Table I.

TABLE I
Effect of Cecectomy on the Growth of Rats
(4 rats in each group)

Group	Diet	Grams gain/week (average 6 weeks)		
		Normal	Cececto- mized	Mock surgery
1	Basal (see text)	30	28	28
2	Basal plus 2% liver extract	33	31	36
3	Basal (duplicate of Group 1)	30	29	
4	Basal plus 100 mg. inositol and 30 mg. <i>p</i> -aminobenzoic acid/100 g.	32	29	
5	Basal plus 0.5% sulfaguandine	17	13	
6	Low B ₁ —100 $\mu\text{g.}$ /100 g.	21	19	
7	Low riboflavin—100 $\mu\text{g.}$ /100 g.	15	13	
8	Low B ₆ —80 $\mu\text{g.}$ /100 g.	22	19	
9	Low pantothenic acid—500 $\mu\text{g.}$ /100 g.	21	19	

The cecectomy technique¹ used in this study and in later experiments was carried out in the following manner. Food was withheld from the animals for 24 hours prior to the operation. The animals were anesthetized with ether and the hair from the abdomen was removed with conc. Na₂S solution. A median incision was made through the skin and abdominal wall which usually brought the cecum into view. The cecum was lifted with a pair of forceps and a ligature was tied around the neck of the cecum at the ileocecal junction in such a way that the opening between the ileum and colon was not obstructed. The cecum was excised close to the ligature and the intestines were then returned to the abdominal cavity without special treatment. The abdominal wall and skin were sutured separately. The operation was performed under clean conditions, but sterile techniques were not employed. The animals recovered rapidly from the operation and growth data obtained with animals merely incised (mock surgery) and sutured in a similar manner, showed that the operation did not appreciably affect the growth as compared to the control animals (Table I).

The subsequent experiments were devised to show the influence of the presence or absence of the cecum on the amounts of thiamine and riboflavin excreted when

¹ We are indebted to Dr. James H. Shaw for assistance in working out the cecectomy technique.

constituents of the diet other than the B vitamins were varied. Since the type of carbohydrate has been shown to have a marked effect on the synthesis of certain B vitamins, Morgan, *et al.* (10) and Mannerling, *et al.* (11), high lactose, sucrose, and dextrin diets were used with and without cecectomy and sulfa drug supplementation.

To eliminate as much variation as possible due to growth, adult male rats (weight 200–250 g.) were used. The animals were divided into groups of eight, and four animals in each group were cecectomized. Two control and two cecectomized rats were fed the basal diet and two control and two cecectomized animals were fed the

TABLE II

Effect of Cecectomy and Sulfasuxidine on Thiamine and Riboflavin Excretions

(All values expressed as micrograms per week.)

Treatment	Riboflavin (intake—210 µg. per week)					Thiamine (intake—175 µg. per week)				
	First week	Second week	Third week	Fourth week	Average	First week	Second week	Third week	Fourth week	Average
High Lactose (ave. of 6 rats in each group)*										
Normal	415	400	495	475	445	40	45	86	77	62
Cecectomy	340	320	330	290	320	27	38	24	27	29
Sulfasuxidine	320	275	280	300	295	28	36	47	72	46
Cecectomy + sulfasuxidine	320	230	255	225	260	23	24	24	44	29
High Sucrose (ave. of 2 rats in each group)										
Normal	175	215	200		195	23	28	24		25
Cecectomy	325	185	240		250	26	17	28		24
Sulfasuxidine	185	140	195		175	22	22	26		23
Cecectomy + sulfasuxidine	175	160	175		170	18	20	21		20
(ave. of 4 rats)* High Dextrin (ave. of 2 rats)										
Normal	300	250	250	285	270	55	11	32		33
Cecectomy	210	220	180	250	215	30	21	32		28
Sulfasuxidine	195	180	150	250	195	25	19	26		23
Cecectomy + sulfasuxidine	150	160	135	225	170	28	23	23		25

* Two rats in each group for each experiment. The lactose experiment was repeated 2 times, and the dextrin-riboflavin experiment was repeated once.

basal diet plus 0.75 per cent sulfasuxidine, which was added at the expense of the entire ration. The composition of the lactose, sucrose, and dextrin basal rations was carbohydrate 67 per cent, casein 24 per cent, corn oil 5 per cent, and salts IV 4 per cent. The complete vitamin supplement which contained thiamine 25 μ g., riboflavin 30 μ g., calcium pantothenate 200 μ g., nicotinic acid 25 μ g., pyridoxin 25 μ g., and choline 10 mg. per 0.1 cc. was given daily by dropper. In this and in all other experiments one drop per week of halibut liver oil was fed.

In preliminary experiments the animals were placed in metabolism cages and the urine and feces collected in dark colored bottles. The excreta which collected on the funnels were washed into the bottles each day to prevent change after voiding.

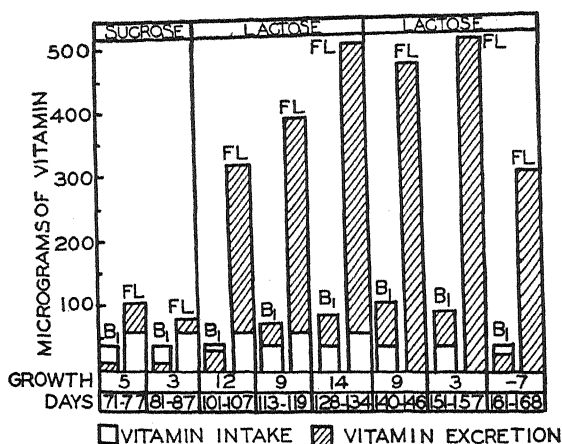


FIG. 1

Effect of Dietary Carbohydrate and Riboflavin on the Excretion of Thiamine and Riboflavin
Average of 6 rats

Toluene and chloroform were added to the receivers. After 2 weeks of habituation, 3-4 weekly collections were made with 4-6 day intervals in between periods of collection. In later experiments a different method of collecting the excreta was used. The urinary and fecal excretions were collected in large glass pans.² Three hundred cc. of 0.5 N H₂SO₄ were added to each pan, and the pan was then placed under the dormitory cages (½ inch mesh screens). The cages were placed in a dark corner of the room and fabric placed over the cages to prevent destruction of the riboflavin. Food and water were given *ad libitum*, and the contents of the water dishes were rinsed into the pans each day and fresh water was supplied.

It was not possible to collect the urine and feces separately, due to the slight diarrhea which resulted when the high lactose diets were fed. At the end of each week

² We wish to thank Mr. L. J. Teply and Mr. W. A. Krehl for this suggestion.

the volume of the excretion sample was measured, and the entire sample was homogenized in a Waring blender. A suitable aliquot was then taken for thiamine and riboflavin determinations. Thiamine was determined by the thiochrome method (12) and riboflavin by the fluorometric method of Conner and Straub (13) as modified by Andrews (14). The average excretion for thiamine and riboflavin per week for all animals in each group over periods from 3-4 weeks under the conditions previously outlined are shown in Table II.

In one experiment subminimal vitamin levels were given, since the beneficial effects due to microbial synthesis should be more evident under these conditions. Six male animals which had received the sucrose basal ration (sucrose 73 per cent, casein 18 per cent, salts IV 4 per cent, and corn oil 5 per cent plus 6 μ g. of thiamine, 8 μ g. of riboflavin, 80 μ g. of calcium pantothenate, 125 μ g. nicotinic acid, 6 μ g. of pyridoxin, and 10 mg. of choline per day by dropper for 12 weeks, were available for this experiment. The average weight of the animals at that time was 125 g. The total excretions were collected for two weekly periods, then the diet was changed to the high lactose diet (lactose 67 per cent, casein 24 per cent, Salts IV 4 per cent, and corn oil 5 per cent) and after a two week orientation period, collections were made for 3 weeks, allowing several days to elapse in between periods of collection. Since the growth rate increased during this period, riboflavin was removed from the supplement and collections made in a similar manner for 3 more weekly test periods. The results are presented graphically in Fig. 1.

DISCUSSION

The studies carried out on the growth of cecectomized young rats, with restrictions in thiamine, riboflavin, calcium pantothenate, and pyridoxin intake, failed to reveal any substantial differences in the growth rate of normal and cecectomized rats. This indicates that the rat is not dependent, to any great extent, on the cecum for the production or absorption of any of these B complex vitamins under our experimental conditions. The rats which received sulfaguanidine showed the widest growth differences between normal and cecectomized groups. This indicates that sulfaguanidine exerted antibacterial action in some other regions of the tract. Day, *et al.* (6) have shown that a severe hypoprothrombinemia occurs when cecectomized rats are fed sulfa drug. Cecectomized rats which did not receive sulfasuxidine developed a mild hypoprothrombinemia. It must be kept in mind that our studies have been carried out only on sucrose rations, perhaps a marked difference would be obtained when other carbohydrates are fed.

Although these growth studies failed to show any appreciable growth differences due to cecectomy, excretion studies carried out with adult rats demonstrated the importance of the cecum in vitamin syntheses, particularly when a high lactose ration was fed. Similar studies with

high sucrose and dextrin diets show somewhat different results (Table II).

The amount of thiamine excreted was only a fraction of the intake on any of the three diets, however cecectomy and sulfasuxidine treatments resulted in a decreased excretion on the high lactose diet. Very similar results were obtained under all conditions when high sucrose or dextrin diets were ingested. It is interesting to note that none of these carbohydrates is favorable for thiamine synthesis under these experimental conditions, while Najjar and Holt (15) have shown dextrimaltose to be favorable for thiamine synthesis in humans.

Approximately twice as much riboflavin was excreted as ingested when a high lactose diet was employed. Sulfasuxidine or cecectomy decreased the riboflavin synthesis considerably; sulfasuxidine and cecectomy together reduced the riboflavin synthesis still more, although the total excretion was never below the riboflavin intake which was 210 μ g. per week. Riboflavin excretions were not appreciably affected by cecectomy or sulfasuxidine when sucrose was the carbohydrate. The excretion of riboflavin observed on dextrin diets was higher than on sucrose, but much lower than on lactose diets. These results are in agreement with the comparative effects of sucrose, lactose, and dextrin on riboflavin production reported by Mannering, *et al.* (11), however these workers used young rats which were receiving a low riboflavin intake. It is of interest that the growth obtained averaged 7 g. per week on lactose and sucrose, and 18 g. per week on dextrin diets. This probably decreased the amount of thiamine and riboflavin excreted on the dextrin regimen. Sulfasuxidine did not markedly retard growth on dextrin rations, but some retardation was noted with sucrose and lactose diets.

It is realized that the amount of any vitamin excreted does not measure true synthesis, since the amounts absorbed or destroyed can not be differentiated. Also any change in weight (anabolism or catabolism) will undoubtedly affect the amount excreted. When the amount excreted is considerably more than the intake over a considerable period of time, there seems little doubt that considerable vitamin synthesis has occurred, but it does not prove that the synthesis has been beneficial to the host.

In the experiment on subminimal B vitamin levels the casein level was raised from 18 to 24 per cent when the animals fed the sucrose diet were given the lactose diet. This change in protein level could

not logically explain the increase in vitamin synthesis which occurred, but it might have caused the increase in growth (Fig. 1). The decrease in growth observed when the riboflavin was removed from the diet indicated that the utilization of the vitamins synthesized on the lactose ration was responsible for the additional growth rather than the increase in the level of casein in the diet.

This experiment shows that lactose serves as a better medium than sucrose for thiamine and riboflavin synthesis. Partial utilization of the synthesized vitamins undoubtedly accounted for the increased growth. When riboflavin was removed from the diet, utilization of these vitamins was apparently not sufficiently great to maintain the same increased rate of growth. The amount of thiamine and riboflavin synthesized in this period dropped considerably, which follows with the decreased growth. These observations could be explained by the fact that the dietary riboflavin and lactose were responsible for supporting a flora capable of synthesizing more thiamine and riboflavin and when the riboflavin was removed, this flora could not be maintained. Due to a decrease in these microorganisms, a decrease in synthesis of thiamine, riboflavin, and possibly other factors occurred. This would also explain the "lag" observed in the amount of thiamine and riboflavin excreted.

SUMMARY

1. Cecectomized rats on complete synthetic rations or synthetic rations which contain limited amounts of the individual B vitamins grew at approximately the same rate as their controls. This indicates that growing rats on sucrose diets do not depend to any extent on the cecum for absorption or synthesis of these known or required factors.

2. The synthesis of riboflavin in the intestinal tract of rats which received lactose as the only carbohydrate, was decreased by cecectomy or by feeding sulfasuxidine. Similar effects could be demonstrated on dextrin diets, but with sucrose diets very little difference due to cecectomy or sulfasuxidine was observed.

3. Lactose, sucrose, and dextrin diets are not conducive to marked thiamine synthesis, only about 25 per cent of the intake could be accounted for in the urine and feces.

4. Rats previously maintained on sucrose diets with suboptimal amounts of the B vitamins showed an increase in growth and a corresponding rise in thiamine and riboflavin excretion when fed lactose

diets. It is believed that some of the synthesized vitamins were utilized by the animal, accounting for the increased growth. When the riboflavin was removed from the diet a reduction in growth and a subsequent reduction in thiamine and riboflavin excretion occurred.

5. The total excretion of riboflavin on lactose diets was approximately the same when either 210 μ g. or 56 μ g. of riboflavin per week were fed. About twice as much riboflavin was excreted as ingested when the high level was fed and nearly ten times as much was excreted when only 56 μ g. per week were fed.

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The Determination of Sulfate by the Benzidine Method

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INTRODUCTION

The estimation of sulfate by the benzidine method is a convenient procedure, but it is subject to error from occlusion and from incomplete precipitation. It has been shown by Fiske (1) that for greatest accuracy phosphates should be removed before precipitating the sulfate and by Owen (2) that the minimum solubility of benzidine sulfate is at pH 2.75. The solubility of benzidine sulfate in water is appreciable, and hence it is customary to wash the precipitate with a saturated solution of benzidine sulfate or with aqueous acetone or alcohol.

Occlusion occurs: if the concentration of benzidine hydrochloride in the precipitating system is too great; if too concentrated a benzidine reagent is used; if the reagent is added too rapidly; or if the system is cooled too rapidly. Precipitation is incomplete if the precipitating system is so dilute that the ion-product of benzidine and sulfate falls below a certain limit or if the time allowed for precipitation is too short. Salts increase occlusion and decrease the rate of precipitation, the heavier ions being most effective, but they do not, as has been suggested, affect appreciably the solubility of benzidine sulfate. Evidently the combined effects of occlusion and incomplete precipitation may neutralize each other and produce apparently correct results. The present experiments have shown (or substantiated) that occlusion may be decreased or eliminated in several ways: by using a dilute benzidine reagent, adding the reagent very slowly, adding it in increments with a pause of several minutes between increments, or by adding the reagent more rapidly or in greater concentration to the system heated to 50–60°C. Furthermore, if sufficient time is allowed for complete precipitation of the sulfate, under appropriate conditions, the occluded salts redissolve.

PROCEDURE

The test material, $M/10$ H_2SO_4 , was neutralized with $NaOH$ using bromphenol blue as the indicator. Tenth normal HCl was then added until the blue color disappeared, then either one or two drops more or 2 ml. M acetate buffer of pH 2.8. The resulting pH is that at which benzidine sulfate is least soluble (2). Salts were added when desired and appropriate dilutions made.

In earlier experiments, a saturated solution of benzidine dihydrochloride (0.22 M) in 0.4 N HCl was used; in later work, this was diluted four-fold. The reagent was added either slowly or rapidly; concentrated or dilute; at room temperature or at 50–60°C. Acetone equivalent to one quarter of the final volume of the mixture (1) was added 3 to 10 minutes later. The systems were allowed to stand from 10 minutes to 24 hours either at room temperature or at 0°C.

After filtering off the benzidine sulfate the flask and precipitate were washed with 2 ml. of 50 per cent aqueous acetone followed by four 2 ml. washes of pure acetone. The precipitate was then washed from the filter with water and titrated hot with 0.02 N $NaOH$ using phenol red as the indicator.

RESULTS

The effect of salts is shown by the data presented graphically in Fig. 1. In these experiments 0.1 mM of sulfate and 0.44 mM of benzidine were used; the reagent was 0.22 M and was added in a rapid

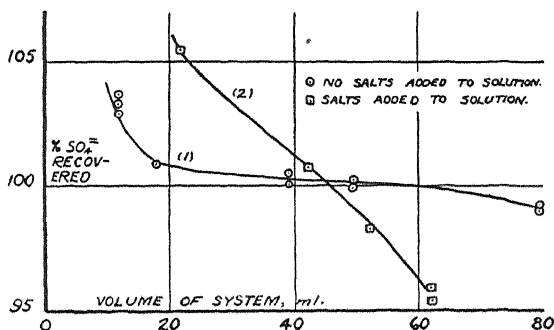


FIG. 1

Effect of Salts on the Precipitation of Benzidine Sulfate

stream of drops; the precipitation time was 10 minutes. The salts used were $NaCl$ (2 mM), $NaClO_4$ (2 mM), and $CuCl_2$ (0.2 mM). In the absence of salts (Curve 1) correct results are obtained over a wide range of concentrations, occlusion occurring only in the most concentrated solutions and incomplete precipitation only in the most dilute. Salts

increase occlusion in the more concentrated solutions and delay precipitation in the more dilute to such an extent that Curve 2 crosses the 100 per cent axis at a steep angle. Further experiments showed that increasing the time of precipitation to 24 hours gave lower values in the more concentrated systems and higher in the more dilute, but the range of concentrations over which correct results were obtained was quite limited. It is interesting that results even greatly in error are quite reproducible.

The dependence of sulfate recovery and occlusion on the time allowed for precipitation and the concentration in the system is shown by the data represented in Fig. 2. These systems contained 0.1 *mM* sulfate

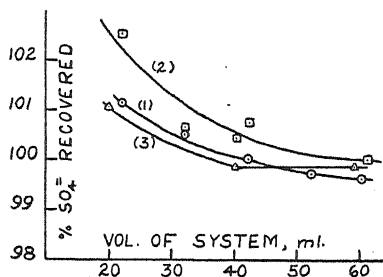


FIG. 2

The Relation of Sulfate Recovery to Time of Precipitation and to the Concentrations in the System

Precipitation times: Curve 1, 10 minutes; Curve 2, 2 hours; Curve 3, 20 hours.

and essentially the same salts as before. The dilute benzidine reagent (0.44 *mM* of 0.044 *M* solution) was added slowly at a rate of 1–3 ml. per minute ($\frac{1}{2}$ to 1 drop per second), at room temperature, with stirring. After 10 minutes the acetone was added and 5–10 minutes later the systems were cooled to 0°C.

The results using a precipitation time of 10 minutes (Curve 1) appear, superficially, to be satisfactory when the volume is greater than 30 ml., but the fact that the 2 hour precipitations (Curve 2) give higher values throughout the range of dilution indicates that, for the shorter time, incomplete precipitation and occlusion must have both occurred, but that at the intermediate concentrations they have neutralized each other. The values for precipitation at 16–23 hours (Curve 3) are lower than the 2 hour values and, except in the most

TABLE I
Sulfate Recovery under Several Conditions

Exp. No.	Total volume ml.	Benzidine mM	Sulfate mM	Ion product benzidine \times sulfate $\times 10^6$	Sulfate recovered per cent	Precipitation time hours
(a) Precipitation of Benzidine Sulfate by the Slow Addition of Dilute Benzidine Reagent						
1	45	1.323	0.0245	1.60	102.0	20
2	50	"	"	1.30	101.5	"
3	60	"	"	0.91	98.5	"
4	80	"	"	0.51	97.5	"
5	35	0.663	0.0507	2.74	100.8	18
6	45	"	"	1.65	100.0	"
7	59	"	"	0.97	100.0	"
8	22	0.442	"	4.62	100.8	19
9	30	"	"	2.49	100.2	23
10	40	"	"	1.40	98.4	19
11	49	"	"	0.93	97.6	18
12	60	"	"	0.62	97.0	19
13	30	0.663	0.0995	1.83	102.4	20
14	40	"	"	4.03	100.6	"
15	50	"	"	2.65	100.0	23
16	60	"	"	1.83	99.6	18
17	20	0.442	"	10.00	101.1	16
18	22	"	"	9.07	100.4	23
19	40	"	"	2.74	99.8	16
20	59	"	"	1.26	99.9	16
(b) Precipitation of Benzidine Sulfate by the Slow Addition of Dilute Benzidine Reagent at 50-60°C.						
21	30	0.663	0.0507	3.74	99.6	20
22	36	"	"	2.75	99.0	"
23	40	"	"	2.11	100.0	"
24	50	"	"	1.34	98.0	"
25	60	"	"	0.93	98.8	"
26	30	"	0.0995	7.34	100.0	"
27	31	"	"	6.88	100.5	"
28	36	"	"	5.39	100.1	"
29	40	"	"	4.13	99.4	"
30	40	"	"	4.13	99.9	"
31	50	"	"	2.64	100.3	"
32	64	"	"	1.61	99.8	"

TABLE I—*Continued*
Sulfate Recovery under Several Conditions

Exp. No.	Total volume ml.	Benzidine mM	Sulfate mM	Ion product benzidine \times sulfate $\times 10^5$	Sulfate recovered per cent	Precipitation time hours
(c) Precipitation of Benzidine Sulfate by the Slow Addition of Dilute Benzidine Reagent in 5 ml. Increments at 5 Minute Intervals						
33	40	0.663	0.0507	2.11	99.8	20
34	60	"	"	0.93	98.6	"
35	80	"	"	0.55	98.4	"
36	30	"	0.0995	7.34	100.2	"
37	50	"	"	2.64	100.1	"
38	80	"	"	1.03	98.4	"

Salts added to systems (mM): Exp. 18, 2 NaCl; Exps. 17, 19, and 20, 2 NaCl + 2 NaClO₄ + 0.2 CuCl₂; in other exs., 2 KCl + 2 NaClO₄ + 0.2 CuCl₂. Unless stated otherwise, the reagents were added at room temperature; precipitation was at 0°C.

concentrated solutions, give sulfate recoveries correct to within 0.5 per cent showing that the occluded material (present at 2 hours) must have dissolved.

The results obtained with mutually varying amounts of sulfate and benzidine at a number of dilutions and under several conditions of precipitation are shown in Table I. In part (a) of the table the procedure was that just described. It gives satisfactory results over a considerable range of concentrations, but when the mutual concentration of sulfate and benzidine is increased beyond certain limits occlusion occurs. These limits may be extended as seen in parts (b) and (c) of Table I either by adding the dilute benzidine in increments with an interval of 5 minutes between increments, or by heating the solution to 50–60°C., under which condition the benzidine may be added rapidly.

The ion-product of sulfate and benzidine has been calculated for those experiments in which the mode of addition of the benzidine and the dilution were such as to prevent occlusion. In Fig. 3 the values are plotted against the sulfate recovery. The graph shows that irrespective of the actual concentration of sulfate or of benzidine, complete precipitation is obtained as long as the ion-product is greater than about 2.0×10^{-5} . This generalization is valid, of course, only within the limitations of the experiments, the most important of which is that

the systems contained 25 per cent acetone; probably of less importance is the salt content of 4 mM.

The approximate amounts of benzidine (shown in parenthesis) to use with the amounts of sulfate indicated, all on a millimolar basis, are: 0.025–0.050 (1.33); 0.050–0.075 (0.66); 0.100 (0.44).

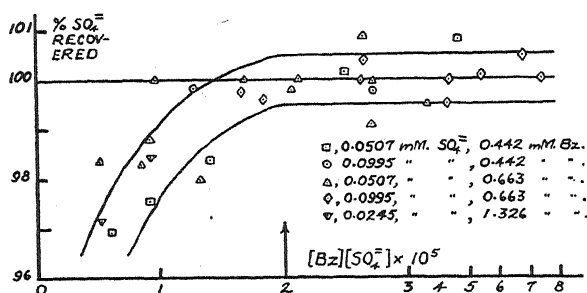


FIG. 3

Relation of Sulfate Recovery to the Ion-product of Benzidine and Sulfate

TABLE II

Recovery of Inorganic Sulfate from Urine by the Modified Benzidine Method and by Fiske's Procedure¹

Exp. No.	Benzidine added		Urine filtrate	Sulfate added (as S)	KCl added	S/ml. urine		Error	Ion product (SO_4) \times (Bz.) $\times 10^6$
	Amount	Volume				Found	Calculated		
	mM	ml.	ml.	mg.	mM	mg.	mg.	per cent	
1	0.88	20	10.01			0.603			15
2	0.44	10	20.02			0.602			15
3	0.88	20	10.01		4	0.606	0.603	+0.5	15
4	0.44	10	20.02		4	0.598	0.603	-0.5	15
5	"	"	10.01	3.298		2.296	2.260	+0.4	142
6	"	5	10.01			0.610	0.603	+1.1	7.5
7	"	"	5.00			0.307	0.302	+1.6	3.8
8	"	"	10.01		4	0.625	0.603	+3.7	7.5
9	"	"	5.00		4	0.295	0.302	-2.0	3.8
10	"	"	"	3.298		2.048	1.959	+4.5	71
11	"	"	"		4	2.060	1.959	+5.2	71

¹ The modified benzidine method was used in exps. 1–5; Fiske's method in exps. 6–11.

In estimating urinary sulfate by the benzidine method, the usual procedure employed is that of Fiske (1). This method gives results that are sufficiently accurate, for most purposes, when the content of sulfate or of salts does not depart too far from normal. These limits may be exceeded under pathological or experimental conditions.

Urines to which extra sulfate and salts were added have been analyzed according to Fiske's procedure and according to the procedure developed herein. The phosphate was removed according to Fiske's procedure before precipitating the sulfate. In the modified procedure, the benzidine was added drop-wise in 5 ml. increments and the mixture maintained at 0°C. for 20 hours. The volume in all cases was 40 ml., but since Fiske used a volume of 16 ml., the amounts of all reagents was increased 2.5 times when using his procedure. The data are given in Table II. Experiments 1 and 2 made according to the modified procedure may be taken as giving the correct value for the urinary sulfate. When the urine contains no added sulfate or salt Fiske's method gives moderate accuracy, but when either of these is present the error may be as much as 5 per cent. The modified procedure applied to the same samples gives results accurate to 0.5 per cent.

SUMMARY

1. In the benzidine method of sulfate analysis, the factors governing occlusion and incomplete precipitation have been investigated.
2. A procedure has been developed that gives results correct to within 0.5 per cent when precipitating 0.025 to 0.1 *mM* sulfate.
3. Under the experimental conditions used, the ion-product of sulfate and benzidine must exceed 2×10^{-5} for complete precipitation of sulfate.
4. In urines containing a high content of sulfate or salt, where Fiske's benzidine method is inapplicable, the present procedure can be used.

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The Constitution of Salmin

I. Amino Acid Composition

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INTRODUCTION

The recent investigations of Mirsky and Pollister (1), Greenstein (2), Stedman (3) and others have renewed interest in the classical studies of Miescher (4) and of Kossel (5) on the genetically important protamines and histones. Since the chemical work of Miescher (4) and of Kossel and Dakin (6), little experimental work has been carried out on salmin as most of the investigations on the protamines by Felix, Waldschmidt-Leitz, Edlbacher and others have been on clupein.

In Kossel and Dakin's (6) 1904 analysis of salmin, arginine was calculated from the quantity of nitrogen precipitated by silver in alkaline solution, while proline was estimated from the amount of nitrogen which was soluble in alcohol. The quantities of serine and aminovaleric acid (valine) were based upon the elementary analysis of the mono amino acid fraction which was insoluble in alcohol. Kossel and Dakin (6) first pointed out that the salmin group of protamines yield approximately two molecules of arginine per molecule of mono amino acid. This has been consistently repeated by subsequent investigators, thus testifying to the remarkable accuracy of these classical studies. Although the arginine content of salmin has been confirmed by Kossel and Gross (7) and others (cf. 5), as far as we are aware, there have not been any analyses for the mono amino acids in salmin using the newer techniques. Such data are reported in this paper as well as a reinterpretation of some of the older studies.

EXPERIMENTAL

A sample of salmin sulfate prepared from Columbia River salmon was obtained from a commercial source where it is used in the manufacture of protamine insulin. The pure white fluffy powder contained 24.46 per cent of nitrogen by the Kjeldahl method employing selenium-copper catalyst and a 6-hour digestion period and 24.2 per cent of nitrogen by the micro Dumas method. The salmin sulfate also contained 19.1 per cent of H_2SO_4 and 0.18 per cent of ash. These values are on the moisture-free material.

Kossel and Dakin (6) indicated the presence of only four amino acids in salmin: arginine, proline, serine, and amino valeric acid. Nevertheless, quantitative determinations were carried out for 15 amino acids, both for the purpose of ascertaining whether the preparation was devoid of non-protamine proteinaceous material such as the "gewebsbildende Gerüst" of Miescher (4) or the chromosomin of Stedman (8) and for the purpose of finding out whether other amino acids are in the salmin molecule.

Arginine, histidine, and lysine were determined by a micro-modification of the Kossel-Kutscher method (cf. 9). Arginine was also determined by direct precipitation with flavianic acid according to Kossel and Gross (7) as modified by Vickery (cf. 9). Lugg's adaptation (cf. 9) of the Millon reaction was used for the estimation of tyrosine and tryptophan, while the Kapeller-Adler reaction (cf. 9) was used for phenylalanine. Both the Folin and Fleming-Vassel methods (cf. 9) were used to detect cystine, while the McCarthy-Sullivan procedure (cf. 9) was used for methionine. The absence of threonine was shown by the Block-Nicolet oxidation method (cf. 9). Serine was determined by the formation of HCHO according to Nicolet and Shinn (cf. 9). Because of Martin and Synge's criticism (10) of the Nicolet-Shinn serine method, the quantity of serine was also calculated from the yield of ammonia in the mono amino acid fraction and in the whole hydrolyzate by the alkaline periodate method of Van Slyke, Hiller, and MacFadyen (cf. 9). Serine determined by these procedures checked within 4 per cent. The Nicolet-Shinn results are used in the table.

Leucine, isoleucine, valine, and glutamic acid were determined by the microbiological method according to Kuiken, *et al.* (cf. 9), Shankman (11), and McMahan and Snell (12). Ten tubes were set up for the unknown and 20 for the standard reference curve for each run. The presence of valine and isoleucine was confirmed by oxidation to acetone and ethylmethylketone respectively (cf. 9).

The sum of proline plus hydroxyproline was estimated by Guest's modification of Lang's pyrrole method (cf. 9) after removal of the greatest part of the arginine by precipitation with silver sulfate and barium hydroxide and also on the entire hydrolyzate. Each unknown solution was read against a closely checking proline standard prepared at the same time and under the same conditions.

Alanine was estimated from the acetaldehyde formed by ceric sulfate oxidation from the lactic acid produced by deamination of the hydrolyzate (cf. 9). The formation of CH_3CHO was confirmed by the isolation of the dinitrophenylhydrazone (m.p. 160°C .).

The pH of salmin sulfate solution in water was 3.1.

RESULTS AND DISCUSSION

The analytical results as well as those of Kossel and Dakin (6) and of Kossel (5) are summarized in Table I.

TABLE I
Amino Acids in Salmin

Amino acid	Salmin sulfate	Salmin		Distribution of nitrogen		
		Author	Kossel-Dakin (6)	Author	Kossel-Dakin (6)	Kossel (5)
Arginine	68.57	88.4	87.4	90.2	89.2	89.3, 90.6
Proline	6.10	7.9	11.0	3.0	4.3	
Serine	5.44	7.0	7.8	3.0	3.3	
Valine	3.2	4.1	4.3	1.6	1.7	
Alanine	2.8	3.6		1.8		
Isoleucine	1.15	1.5		0.5		
Histidine	0					
Lysine	0					
Tyrosine	0					
Tryptophan	0					
Phenylalanine	0					
Cystine	0					
Methionine	0					
Leucine	0					
Glutamic Acid	0					
Sulfuric Acid	19.1					
Sum of Amino Acids		112		100.1		

The absence of histidine, lysine, glutamic acid, the aromatic and the sulfur-containing amino acids indicates the absence of extraneous proteins in the salmin sulfate preparation.

The values for arginine, serine, and valine are in good agreement with those of Kossel and Dakin (6). Our values for serine are somewhat lower while those for proline are definitely lower than the earlier figures. These differences may well be explained by differences in method.

Hydroxyproline may be present in salmin as the Guest method is not specific for proline alone. It should be recalled that Felix and

Mager (13) demonstrated that clupein contains hydroxyproline. The possible presence of this amino acid in salmin will be the subject of a future investigation.¹

Table II gives some calculations derived from the amino acid values.

TABLE II

Molecular Ratios, Nitrogen Distribution, and Molecular Weight of Salmin

Amino acid	Molecular ratio	N atoms	Molecular weight
Arginine	47 ± 1	188 ± 4	
Proline	6	6	8785
Serine	6	6	9005
Alanine	4	4	9896
Valine	3	3	8565
Isoleucine	1	1	8858
Average			9022*

* Molecular weight from elementary composition, 9100.

The minimum molecular weight calculated from the percentage composition of isoleucine (×1), valine (×3), alanine (×4), proline (×6), and serine (×6) is approximately 9300; while the molecular weight, calculated from the sum of the residue weights, is approximately 9100.

Felix and co-workers (cf. 13) have shown that clupein, as obtained by their rather drastic procedures, is usually a mixture of several molecular species, the larger component having a minimum molecular weight of approximately 4500. It may be, therefore, that this preparation is composed of two components, one with and the other without isoleucine.

The data suggest the following formulae for salmin sulfate:



where A represents arginine, P proline or hydroxyproline, S serine, aL alanine, V valine, and I isoleucine.

Kossel (5) suggested that monoprotonamines of the salmin type are characterized by having two molecules of arginine per mole of mono amino acid, which he formulated as given below; although components containing as many as four arginine residues per mono amino acid were also mentioned by him.

¹ Repeated tests for hydroxyproline by the peroxide method of McFarlane and Guest (cf. 9) indicate that this amino acid is not present in salmin.

I. AAM-AAM-AAM---- or II. AMA-AMA-AMA----

Formula I is supported by the experiments of Felix (13, cf. 2) who was able to isolate arginylarginine from clupein and of Waldschmidt-Leitz and Kofranyi (14) who isolated two dipeptides of the structure AM and MA and three tripeptides having the structure AMA. Felix and Dirr (15) suggested, but did not prove, that the terminal carboxyl group of clupein belongs to arginine and that proline is the first mono amino acid in the chain. Kossel (5) also reported the absence of formal titratable N in salmin. On the other hand, Waldschmidt-Leitz (16) claims that, although the exact position of proline in clupein is not known, it is not the terminal mono amino acid.

The data obtained in this study indicate that the ratio of arginine to mono amino acids is not 2:1, but somewhat higher. A tripeptide of two arginine residues and a mono amino acid (AAM) would have 88.8 per cent of its nitrogen as arginine. A tetrapeptide of three arginine residues to one mono amino acid would have 92.3 per cent of its N as arginine N. The nitrogen distribution figures of Kossel and Dakin (6), Kossel and Gross (7), and Kossel and Staudt (cf. 5) vary from 89.2 to 90.6 per cent if the arginine N is calculated on the basis of the total N before removal of sulfuric acid while the per cent of arginine N may be as high as 93.4 per cent when it is calculated from the total N after removal of the H_2SO_4 . The value of 90.2 per cent given in Table I is calculated from the yield of pure arginine flavianate and the nitrogen in the intact protamine.

These results suggest that the ratio of mono amino acids to arginine is not 1:2, but that some extra arginine is present. The value 90.2 per cent arginine N of total N would be accounted for by the presence of one AAAM group for every two AAM peptides. Naturally, if AM and AAAAM peptides are assumed, the experimental results could be also explained.

Using the above information, the following very tentative structure may be written for salmin as a working hypothesis for further investigations. The location of the mono amino acids along the chain is purely speculative.



SUMMARY

The presence of 88 per cent of arginine, 7.9 per cent of proline, 7.0 per cent of serine, 4.1 per cent of valine, 3.6 per cent of alanine, and 1.5 per cent of isoleucine accounts for the nitrogen of salmin.

The absence of histidine, lysine, tyrosine, tryptophan, phenylalanine, cystine, methionine, and glutamic acid is demonstrated. Leucine could not be found by the microbiological method.

The presence of alanine in salmin and of isoleucine in a mono protamine is shown for the first time.

We are indebted to Mr. Everett H. Lang, Burroughs Wellcome & Co., for the micro Dumas determination.

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Studies on the Biochemistry of *Tetrahymena*

I. Amino Acid Requirements

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INTRODUCTION

A number of attempts have been unsuccessful in this and other laboratories (1, 2, 3, 4) to obtain growth of the ciliate *Tetrahymena geleii* in media where the sole source of nitrogen is represented by amino acids. This failure has made it impossible to carry out critical studies on the growth factors required, except where protein or peptone base media could be treated in such a way as to render them free of single known substances (5, 6). Recently it has been found possible to obtain growth in completely hydrolyzed proteins (6), and previous failures can now be traced to lack of suitable supplementary substances and not to a specific peptide requirement, as proposed by Lwoff (1). The purpose of this paper is to report our findings on the specific amino acid requirements of *T. geleii* W and to describe the conditions under which these ciliates can be grown in a basal medium of known chemical composition.

EXPERIMENTAL

The ciliated protozoon *Tetrahymena geleii* W was used in this study. This strain has been maintained in this laboratory in pure culture (bacteria-free) for a number of years, and has been used in several of our previous investigations (4, 5, 6, 7).

Our studies have shown that growth is impossible (even with the addition of eleven known factors) in the absence of certain unknown substances which we have obtained in protein-free form from plant sources. For the purpose of this report we will refer to these substances as "unknown factors."

Unknown Factors. Cerophyl² (100 g.) was suspended in 1 liter of distilled water

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² Cerophyl Laboratories, Kansas City, Mo.

and 10 per cent sulfuric acid added to give a pH of 2.0-3.0. After autoclaving at 15 lbs. pressure for 15 min. the solid material was filtered off with suction using Standard Super Cel³ as a filter aid. This extract was then neutralized with sodium hydroxide and the precipitate removed by filtration. The extract was then sterilized by autoclaving.

To obtain the protein-free preparation used in these studies, the extract was again adjusted to pH 2.0-3.0 with 10 per cent sulfuric acid. The precipitate formed upon the addition of acid was filtered off and discarded. Norit A⁴ was added to the filtrate (2.5 g. per 100 ml.) and the mixture stirred constantly for 30 min. at 60°C. The Norit was then filtered off and washed with 20 ml. of 50 per cent ethanol per gram of Norit. Both the filtrate and the washings were discarded. The Norit was then eluted for 30 min. at 65°C. with a mixture of ammonia, ethanol, and water (1:5:4)

TABLE I
Composition of Amino Acid Medium

Amino acid	mg. %	Known growth factor	γ %
<i>dl</i> -Alanine ⁵	43.5	Biotin methyl ester ⁷	0.005
<i>l</i> (+)-Arginine monohydrochloride ⁵	41.0	Calcium pantothenate ⁷	10.00
<i>dl</i> -Aspartic acid ⁵	17.0	Nicotinic acid ⁴	10.00
<i>l</i> (-)-Cystine ⁵	1.0	<i>i</i> -Inositol ⁴	100.00
<i>dl</i> -Glutamic acid ⁵	25.0	Choline ⁵	100.00
Glycine ⁵	127.0	<i>p</i> -Aminobenzoic acid ⁴	10.00
<i>l</i> (+)-Histidine monohydrochloride ⁴	5.0	Pyridoxin hydrochloride ⁸	10.00
<i>l</i> (-)-Hydroxyproline ⁵	10.0	Uracil ⁴	10.00
<i>dl</i> -Isoleucine ⁵	17.5	Folic acid concentrate ⁹	0.10
<i>l</i> (-)-Leucine ⁵	17.5	Riboflavin ⁷	10.00
<i>dl</i> -Lysine monohydrochloride ⁴	30.0	Thiamine hydrochloride ⁸	10.00
<i>dl</i> -Methionine ⁵	17.0		
<i>dl</i> -Phenylalanine ⁵	7.0	Inorganic salts ¹⁰	mg. %
<i>l</i> (-)-Proline ⁴	47.5	MgSO ₄ ·7H ₂ O	10.00
<i>dl</i> -Serine ⁵	2.0	K ₂ HPO ₄	10.00
<i>dl</i> -Threonine ⁵	10.0	CaCl ₂ ·2H ₂ O	5.00
<i>l</i> (-)-Tryptophan ⁵	5.0	FeCl ₃ ·6H ₂ O	0.125
<i>l</i> (-)-Tyrosine ⁵	10.0	MnCl ₂ ·4H ₂ O	0.005
<i>dl</i> -Valine ⁵	10.0	ZnCl ₂	0.005

To the above was added 200.00 mg. per cent dextrose and 1 part of the "Unknown Factors" in 10 parts of final medium.

³ Johns Manville.

⁴ Eastman Kodak Co.

⁵ Merck and Co.

⁶ Hoffman-LaRoche Co.

⁷ General Biochemicals, Inc.

⁸ Lederle Laboratories, Inc.

⁹ Furnished through the courtesy of Dr. R. J. Williams.

¹⁰ Baker and Adamson.

using 20 ml. per gram of Norit. The elution was repeated once and the eluates combined and reduced *in vacuo* to remove ammonia and ethanol. The eluate was then made up to the original volume of extract used. This eluate was negative to the ninhydrin reaction.

Complete Amino Acid Medium. The amino acids were dissolved separately so that the additions of equal volumes of each would result in the required final concentrations. The concentrations were originally patterned after a 0.5 per cent solution of gelatin. Tryptophan, threonine, and valine were added in arbitrarily chosen concentrations. The final concentrations of the amino acids, known factors (used to insure against any one of these proving to be a limiting factor, rather than a knowledge of their actual requirement, with the exception of thiamine (6)), "unknown factors," dextrose, and inorganic salts are given in Table I.

All media were made with water twice distilled over permanganate in an all-Pyrex still. In the earlier experiments the media were tubed in 5 ml. amounts but later this was reduced to 2 ml. volumes. All media were adjusted to give a pH value of 6.8–6.9 after sterilization by autoclaving.

Growth (population densities) in the experimental series was determined after 72 hours incubation by the direct counting technique (8). In all cases three or more serial transplants were made with loop inoculations (approximately 0.005 ml.). This effectively reduces to an insignificant amount any chemical contamination by carry-over. Cultures were incubated at 25.5°C. The ciliates have been growing continuously in amino acid based media for the past eight months, and the experiments reported here have been repeated eighteen times.

RESULTS

Following the discovery that *T. geleii* W could be grown in completely hydrolyzed casein or gelatin media, provided adequate growth factors were present (6) it was found possible to replace the hydrolyzates with a mixture of nineteen amino acids. Each of the amino acids was eliminated one by one from the mixture with the results shown in Table II. Growth failed when *histidine*, *isoleucine*, *leucine*, *lysine*, *threonine*, or *valine* was omitted from the medium. Barely transplantable growth occurred when *methionine*, *phenylalanine*, or *tryptophan* was omitted. Methionine and phenylalanine were shown to be indispensable for growth by using *dl*-leucine in the mixture. When this was done transplants in media without methionine or phenylalanine were impossible. This shows that traces of these two amino acids were present in the samples of *l*(–)-leucine used. Some tryptophan was present in the natural leucine as growth was reduced from 300 ciliates per ml. to 40 ciliates per ml. when the synthetic leucine was used and tryptophan omitted. Minute amounts of tryptophan must be present in other of the natural amino acids used to account for even this low

TABLE II
Effect of Omitting Single Amino Acids

Amino acid omitted	Trans-plant	Population cells/ml.	Amino acids omitted	Trans-plant	Population cells/ml.
None	1	275,000	<i>l</i> (-)-Leucine	1	5,100
	2	330,000		2	40
	3	320,000		3	0
<i>dl</i> -Alanine	1	210,000	<i>dl</i> -Lysine monohydrochloride	1	16,000
	2	290,000		2	45
	3	270,000		3	0
<i>l</i> (+)-Arginine monohydrochloride	1	61,000	<i>dl</i> -Methionine	1	23,000
	2	7,500		2	100
	3	6,500		3	15
<i>dl</i> -Aspartic acid	1	281,000	<i>dl</i> -Phenylalanine	1	10,000
	2	302,000		2	80
	3	290,000		3	20
<i>l</i> (-)-Cystine	1	230,000	<i>l</i> (-)-Proline	1	213,000
	2	276,000		2	200,000
	3	240,000		3	210,000
<i>dl</i> -Glutamic acid	1	215,000	<i>dl</i> -Serine	1	251,000
	2	220,000		2	266,000
	3	200,000		3	300,000
Glycine	1	11,500	<i>dl</i> -Threonine	1	2,100
	2	190		2	0
	3	260		3	0
<i>l</i> (+)-Histidine monohydrochloride	1	13,000	<i>l</i> (-)-Tryptophan	1	12,000
	2	120		2	350
	3	0		3	300
<i>l</i> (-)-Hydroxyproline	1	258,000	<i>l</i> (-)-Tyrosine	1	260,000
	2	210,000		2	280,000
	3	240,000		3	210,000
<i>dl</i> -Isoleucine	1	6,300	<i>dl</i> -Valine	1	4,300
	2	20		2	30
	3	0		3	0

growth, however, as tryptophan must be added to acid-hydrolyzed Eastman gelatin for transplantable growth.

The omission of arginine reduced growth very markedly and growth failed without it when only the nine indispensable amino acids were present. Arginine, therefore, should be classed as an amino acid necessary for good growth but which the ciliates can synthesize slowly when certain (as yet undetermined) dispensable amino acids are present.

The omission of glycine from the amino acid mixture brought about a sharp drop in growth. These cultures were transplantable, however, at a very low level. It will be noted from Table II that eighteen amino acids (lacking glycine) gave much poorer growth than the minimum ten amino acids (also lacking glycine) (Table III). It appears, then,

TABLE III
Growth in Media Composed of the Minimum Ten Amino Acids With and Without Further Additions

Trans-plant	Minimum ten*	Ten plus 127 mg. % glycine	Ten plus 4 mg. % <i>dl</i> -serine	Ten plus both glycine and serine
1	92,000	160,000	155,000	154,000
2	26,000	84,000	120,000	210,000
3	27,000	89,000	93,000	180,000

* Arginine—82 mg. %; Histidine—10 mg. %; Isoleucine—35 mg. %; Leucine—35 mg. %; Lysine—60 mg. %; Methionine—34 mg. %; Phenylalanine—14 mg. %; Threonine—20 mg. %; Tryptophan—10 mg. %; Valine—20 mg. %.

that one of the main functions of glycine under these conditions is to act as a detoxifying agent for one or more of the dispensable amino acids.

When a mixture of the nine indispensable amino acids and arginine is used fair growth is maintained indefinitely, while the omission of any one results in immediate failure. The concentrations of the ten amino acids was doubled so that the nitrogen content would approximate that of the mixture of the nineteen amino acids. It is apparent that the synthesis of protoplasm is accelerated by some or all of the dispensable amino acids. The addition of glycine to the minimum ten amino acids results in somewhat better growth, and the addition of serine has a more marked effect. The serine effect may appear surprising in view of the fact that serine omission from the complete amino acid mixture had no significant effect. In earlier experiments where sub-optimal growth factors were used the omission of serine did

result in decreased growth. When both glycine and serine are present with the minimum ten amino acids the growth is brought to within slightly less than one division of that obtained in the mixture of nineteen amino acids. The total nitrogen is somewhat higher here, due to the rather large doses of glycine used, and this may account for the increased growth, rather than a specific effect due to glycine. These results are presented in Table III.

DISCUSSION

In an earlier investigation in this laboratory (4) it was found that *Tetrahymena geleii* W could not be maintained in media where the sole source of nitrogen was represented by either acid hydrolyzed casein or a mixture of the ten amino acids found to be essential for the mammal (9). The failure with the hydrolyzate was due to the destruction of tryptophan by the acid during treatment, for we later found (6) that casein hydrolyzate supplemented with tryptophan was satisfactory as a nitrogen source. Failure with the ten amino acid mixture was due to the lack of an essential growth factor in the timothy extract supplements used. Timothy hay extract lacks at least one factor (or contains it in subminimal quantities) which is present in alfalfa or cerophyl extracts and also in many proteins of animal origin. Without this factor growth of *T. geleii* W fails. A report on this factor will appear subsequently. We have found that timothy extract can be substituted for alfalfa or cerophyl extracts as a source of supplementary factors for protein hydrolyzates, provided tryptophan is added.

The toxic effects previously ascribed to amino acids (isoleucine, tyrosine, valine) (4) now appear to be attributable to a sub-optimal concentration of the "unknown" growth factors.

An apparent discrepancy between the present report and one given earlier (6) regarding growth in gelatin and gelatin hydrolyzate to which tryptophan (but no valine) was added can now be explained by valine contamination of the gelatin used (Harris). Eastman de-ashed gelatin fails to support growth under the conditions stipulated unless both tryptophan and valine are added.

The report of Kline (10) on the amino acid requirement of another strain of *Tetrahymena geleii* (*Colpidium striatum*) is difficult to understand in the light of our investigations. He stated that fifteen amino acids were indispensable to his strain but these did not include histidine, leucine, lysine, or threonine. Unless his strain was entirely differ-

ent from our strain or unless it was not bacteria-free it is hard to see how he obtained transplantable growth, even with all nineteen amino acids, in the absence of the "unknown" growth factors. His mixture has consistently failed to support growth of our strain. Unfortunately his strain is no longer available for testing.

In a recent preliminary report (11) it was stated that *Tetrahymena geleii* W did not require isoleucine, phenylalanine, or methionine. It was found, however, that the early failure to demonstrate the indispensability of these amino acids was due to the use of a few amino acids containing traces of the three mentioned. This was particularly noticeable with our sample of *dl*-leucine which contained sufficient isoleucine to mask any requirement for the latter. It became necessary, then, to use *l*(-)-leucine to show an isoleucine requirement, and *dl*-leucine to preclude the introduction of traces of methionine, phenylalanine, and tryptophan. This possibility was suggested by the work of Hegsted and Wardwell (12) with *Lactobacillus arabinosus*, and our results corroborate their findings. Our first sample of *l*(+)-glutamic acid was found to be contaminated with appreciable amounts of isoleucine, phenylalanine, and methionine and was therefore replaced with the synthetic product.

The identity between the amino acid requirements of *Tetrahymena geleii* W and that of the rat (9) and the dog (13) may be of fundamental importance. Even in the case of arginine, the synthetic ability of the ciliate corresponds to that of the young rat. A certain degree of growth (in the ciliate this is represented by population, and therefore cell increase) is possible in the absence of arginine but rate of synthesis of arginine is far below the optimum. The rate of arginine synthesis in the chick, however, is even lower than in the young rat or the ciliate, as maintenance is impossible on an arginine-free diet (14). At first glance (Table II) it would appear that *T. geleii* W resembles the chick (15, 16, 17) in its response to glycine omissions, but it seems probable that the function of glycine in the ciliate is as a detoxifying agent for some dispensable amino acid, rather than a dietary requirement.

Specific amino acid requirements for other microorganisms show either a lower requirement, complete synthetic ability, or in the case of *Lactobacillus arabinosus* (18) a like number (ten). Of these ten there are only seven for which there is a common requirement by the ciliate and the bacterium.

SUMMARY

The single omission of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, or valine from an adequate medium containing nineteen amino acids prevents the growth of *Tetrahymena geleii* W. Omission of arginine and glycine reduces growth markedly. Growth is not possible when only the nine indispensable amino acids are present but the addition of arginine results in fair population densities. Glycine appears to play the role of a detoxifying agent for some dispensable amino acid or acids. Serine is a stimulatory amino acid. The typically animal nature of the amino acid requirements of this ciliate is discussed.

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Studies on the Biochemistry of *Tetrahymena*

II. Factor Three

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INTRODUCTION

The establishment of a basal medium of known chemical composition for the growth of *Tetrahymena geleii* W (1) has made possible a more complete investigation of the growth factor requirements of this ciliate. Evidence has been presented (2) for the requirement of two factors (factor I and factor II) of unknown composition. It is our purpose to present evidence that a third "unknown" factor is required for growth.

EXPERIMENTAL

Tetrahymena geleii W was the organism employed. In testing its response to the various growth factor preparations to be described, two types of basal media were used. The first of these was a mixture of the nineteen amino acids and eleven known growth factors used by Kidder and Dewey (1). The second consisted of a 0.5 per cent gelatin (Harris) hydrolyzate plus tryptophan and growth factors, such as was used in a previous study (3). Control cultures were carried in these basal media plus a hot water extract of cerophyl.²

The following preparations were tested for their effects on the growth of *Tetrahymena*.

1. An extract was prepared by boiling for 15 min. 10 g. of timothy hay per 100 ml. of water, then filtering.

2. Two fractions were prepared by dialyzing a water extract of cerophyl in cellophane tubing against distilled water for 72 hours at 75°C. The fluid outside the membrane was changed at 12 hour intervals. A small amount of hydrochloric acid was added to the original extract and dialysis was considered to be complete when the

¹ Aided by grants from the Morgan Edwards Fellowship Fund and the Manufacturers Research Fund for Bacteriology and Protozoology of Brown University.

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diffusate³ no longer gave a positive test for chloride. The diffusates were combined and their volume reduced to that of the original extract.

A similar process was carried out on the tryptic digest of cerophyl described below.

3. A tryptic digest of cerophyl was prepared by adding 0.5 g. of trypsin (Parke-Davis) to 100 g. of cerophyl in 1 liter of water. After adjusting to pH 8.2, the suspension was incubated under toluene at 37°C. for 36 hours. The mixture was then re-adjusted to pH 8.2 and another 0.5 g. of trypsin added and again incubated for 36 hours. The solid material was then filtered off through a pad of Standard Super Cel and the digest boiled to remove the toluene.

4. An acid extract of cerophyl was prepared by adjusting a mixture of 100 g. of cerophyl in 1 liter of water to pH 2.0-3.0 with sulfuric acid and autoclaving at 15 lbs. pressure for 15 min. (7). The resulting extract was filtered through Standard Super Cel, neutralized with sodium hydroxide and re-filtered.

5. A filtrate of gelatin hydrolyzate (Harris, vitamin-free) was prepared by adding to each 100 ml. of 1 per cent hydrolyzate at pH 2.0, 1 g. of Norit A. This mixture was stirred constantly for 1 hour at 65°C., then filtered through Standard Super Cel and the Norit discarded.

6. An eluate of alfalfa meal⁴ extract was prepared by adsorbing 500 ml. of extract (prepared in the same manner as the cerophyl extract, 100 g. per liter of water) at pH 3.0 with 20 g. of Super Filtrol for 1 hour at 70°C. The Super Filtrol was discarded and the filtrate then adsorbed with 5.0 g. of Norit A. The Norit was then eluted with 100 ml. of ammoniacal ethanol (3 parts ammonia, 5 parts ethanol, 2 parts water). The elution was repeated and the combined eluates were heated to remove the ammonia and ethanol and the volume made up to the original with water. The adsorption and elution were repeated twice more.

These preparations, with the exception of the Norit-adsorbed gelatin, were used to supplement the two basal media described above, in concentrations of 1 part supplement to 9 parts medium. The organisms were carried through at least three transplants in any given medium where growth occurred. Only the results of the third transplant will be reported. Transplants were made at four day intervals using a bacteriological loop. Population density was used as an index of the activity of the preparations. It is recorded as zero to four plus, where four plus represents a population density in excess of 200,000 organisms per ml.

³ The term "dialyzate" has been used to designate the material passing through the membrane (4) as well as the material retained within the membrane (2, 5, 6) (many other examples of both could be quoted). While both usages are correct according to Webster's Unabridged Dictionary, confusion results. The term "diffusate" has been used (2, 6) to refer to the material passing through the membrane, and it is proposed that this term be retained and that the term "non-diffusate" be used to refer to the material remaining within the membrane.

⁴ Thanks are due to A. E. Wilson and the Denver Alfalfa Milling and Products Co., Lexington, Neb., for samples of alfalfa meal.

RESULTS

It may be seen by reference to Table I that growth in a mixture of amino acids occurs only when cerophyl extract or certain fractions of it are present. On the other hand timothy hay extract permits growth of moderate density when hydrolyzed gelatin is used as a source of nitrogen. When cerophyl diffusate is used as a supplement to the basal media the results are similar to those with timothy extract. It is

TABLE I

Supplements	Base media	
	Nineteen amino acids	Gelatin hydrolyzate plus tryptophan
Timothy hay extract	0	+++
Cerophyl extract	+++	++++
Cerophyl diffusate	0	+++
Cerophyl non-diffusate	++	++
Cerophyl diffusate plus non-diffusate	+++	++++
Tryptic digest of cerophyl	++++	++++
Tryptic digest diffusate	++++	++++
Tryptic digest non-diffusate	±	+
Acid extract of cerophyl	++++	++++

therefore apparent that there is some substance present in both gelatin hydrolyzate and in cerophyl which is necessary for the growth of *Tetrahymena*. This substance is lacking in timothy hay extracts.

It will also be noted that in water extracts of cerophyl the substance in question occurs in non-dialyzable form. On the other hand, the active material is found in the diffusates obtained by dialysis of a tryptic digest of cerophyl. The increased growth obtained with such digests may be attributable in part to the addition of growth-promoting material with the trypsin. This is not the entire explanation, however, since extraction of the cerophyl with acid gives a product containing a higher concentration of the growth substance. This may be due to release of the material or to alteration of its structure (7).

TABLE II

Base media	Supplements	
	Alfalfa extract	Alfalfa eluate
Gelatin hydrolyzate plus tryptophan	++++	+++
Norit-treated gelatin hydrolyzate plus tryptophan	0	0
Norit-treated gelatin hydrolyzate plus nineteen amino acids	+++	0

A third line of evidence for the presence of a growth factor in gelatin hydrolyzates is presented in Table II. It is apparent that Norit removes both amino acids and growth-promoting material from the hydrolyzate. However, the removal of amino acids by adsorption is not solely responsible for the lack of growth in this medium. These data also show that there has been a partial removal of this growth factor in the preparation of the alfalfa eluate, by adsorption upon the Super Filtrol.

DISCUSSION

Since two factors (I and II) described as being present in timothy hay extracts (2) have already been proposed, the material present in gelatin hydrolyzate and in cerophyl extract represents a third factor required for the growth of *Tetrahymena*. It may now be stated with certainty that both factors I and II, as well as factor III, are absolute growth requirements for *Tetrahymena geleii* W, since no growth occurs in amino acid mixtures or in gelatin hydrolyzate in their absence (1). The slow growth in casein or gelatin without added supplements (2) must be attributed to a contamination of these proteins with all three factors. Indeed, a nearly optimum concentration of factor III must be present. All the available evidence points to the occurrence of most of factor III in combination with protein. It is released or made dialyzable by digestion of the protein either with acid or enzymatically.

In certain respects, factor III resembles factor B, proposed by Peterson (6) as a requirement for the growth of the ciliate *Colpidium campylum*. If they are identical there must be a large quantitative difference between the requirement of *T. geleii* and that of *C. campylum* for this factor, since Peterson states that extracts of grasses are inadequate as a source of factor B. It is worthy to note, however, that water extracts of cerophyl do not contain an optimum concentration of factor III even for the growth of *T. geleii*.

In its resistance to prolonged heating with concentrated acid, as in the hydrolysis of protein, factor III resembles the "tomato eluate factor" of Kuiken, *et al.* (8). These authors state that their factor merely stimulates the growth of *Lactobacillus arabinosus* 17:5; it is not a growth requirement.

Factor III cannot be identified with any of the factors of known chemical constitution.

SUMMARY

Evidence is presented for the occurrence of a third growth factor for *Tetrahymena geleii*. This factor is present in extracts of cerophyl and alfalfa and also in gelatin. It is stable to heat and strong acid.

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Coupling between Phosphorylation and Oxidation of the 4-Carbon Acids in Rat Kidney Homogenates *

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INTRODUCTION

Earlier papers in this series presented the methods for studying experimental shock in rats (1) and the results of blood and tissue analyses (2, 3) on normal and shocked rats. The general conclusion from those studies was that the mechanisms for the maintenance of the reservoirs of phosphate-bond energy had been seriously impaired in the shocked animals, and it appeared likely that the repercussions of this situation would include a breakdown of the supplies of various biocatalysts needed for the efficient utilization of oxidizable substrates. The biocatalysts include not only the phosphorylated coenzymes, whose functions are fairly well known, but also the protein components of the holoenzymes. In addition, a deficiency of one or more of the hormones such as insulin, adrenalin, thyroxin, and the hormones of the adrenal cortex might develop if energy for their continued production were not available. The correction of the failing metabolism which characterizes shock is readily effected by a restoration of the blood volume if therapy is instituted early, but after a certain point (irreversible stage), blood substitutes are ineffective. We assumed that the loss of one or more replaceable biocatalysts occurs at this stage and that their loss precedes the structural breakdown which would signify a truly irreversible stage of shock which must terminate in death. Although the direct replacement of the hormones listed is possible, it is not feasible to attempt to supply reservoirs of phosphate-bond energy, phosphorylated coenzymes, or apo-enzymes to the cells,

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Wisconsin.

since each cell seems obligated to provide its own energy supply for the synthesis of these biocatalysts from smaller building blocks.

On this basis, one might think that if some fuel whose combustion involved no special phosphorylated coenzymes or hormonal factors could be supplied, it might provide energy for the restitution of the more complex biocatalysts, and thereby restore the total metabolism to its original efficiency. Such therapy might advantageously be used to supplement blood substitutes and might conceivably obviate their use in certain circumstances.

Among the known oxidizable substrates, succinate was the obvious choice, in the light of available information (4). Its oxidation requires fewer cofactors (5, 6) than any other carbohydrate intermediate and it appears to be a normal metabolite. Furthermore, its oxidation has been reported by several investigators to result in the formation of phosphate-bond energy (7, 8, 9, 10). These considerations were put to practical use by Soskin and Taubenhaus (11) who reported the use of sodium succinate to treat nembutal poisoning, and by Mylon, *et al.* (12) who used sodium succinate to treat shock. Although both groups reported considerable success, the case for succinate was weakened by contrary reports on nembutal poisoning (13, 14) and by the finding that sodium succinate was no more effective in shock than an equivalent amount of sodium bicarbonate (15). This latter finding suggests that sodium succinate is effective only when it can be completely oxidized and indicates that succinate is no better than any other intermediary metabolite. However, Mylon, *et al.* (16) have obtained positive therapeutic effects with succinate and fumarate, which appeared to be unrelated to the correction of acidosis. It appears possible that under some circumstances the concentration of four-carbon dicarboxylic acids might be a limiting factor in other oxidations but it is clear that the nature of the energetic coupling which follows succinate oxidation is still but vaguely understood. The fact that succinate could be oxidized by tissue from shocked animals (4, 17) seemed to intensify the paradox as to why succinate was less successful in the treatment of shock than had been anticipated, and suggested that the existing observations were either wrong or incomplete. The latter seems to have been the case. Shorr (4) reported that in tissues which had been hypoxic, succinate oxidation did not yield ATP, and further that the oxidative decarboxylations were diminished during succinate oxidation.

It was apparent that a more intimate knowledge of the factors which affect the yield of phosphate-bond energy from tissue oxidations was needed. The succinate system was chosen as the first point of attack, and the results of this study will be reported here.

EXPERIMENTAL

Previous work on oxidative phosphorylation (7, 8, 9, 10, 10a) has been carried out using washed minces or concentrated dialyzed cell-free

extracts equivalent to several hundred milligrams of tissue in 2 or 3 ml. final volume. Experience with homogenates has shown that succinate oxidation can be studied with extremely low tissue concentrations provided the reaction mixture is fortified with cytochrome *c* (5, 6). Similarly, potassium ions were found to be present in too low a concentration for transphosphorylation in highly diluted homogenates (18). By adding all of the soluble components at concentrations suggested by the references cited, it was possible to couple phosphate esterification with the oxidation of the C_4 acids, using only 20 mg. of homogenized fresh rat kidney per 3 ml. final volume. At this dilution, the preparation does not need to be dialyzed in order to demonstrate the action of the various added components. Furthermore, the use of homogenates makes it possible to minimize the breakdown of some of the biocatalysts which are extremely labile.

METHODS

The conditions which were used for the study of succinate oxidation will be described first. Succinic acid is the first C_4 acid which arises in the course of glucose oxidation according to the Krebs cycle, and its oxidation requires fewer soluble cofactors than any other substrate in the cycle. According to present data, cytochrome *c* is the only soluble co-factor in the succinoxidase system. We assumed *a priori* that (1) the coordination of the several enzymes involved in the reactions to be studied would require that all factors be present in nearly optimum amount; (2) delayed substrate addition would be unsuccessful; (3) speed and the use of cold would be essential; (4) adenosinetriphosphate would be the compound of choice for the phosphate transmitting system; (5) creatine would be the best phosphate acceptor; (6) the time of incubation should be brief; and (7) cytochrome *c*, plus magnesium, inorganic phosphate, fluoride, chloride (19), sodium and potassium ions would have to be present in the system, in addition to tissue and substrate. With these considerations in mind, phosphate esterification was readily accomplished, and the validity of the various assumptions was then tested one at a time.

The procedure is as follows: Warburg flasks with no side-arms are used. Thirty-milligram portions of creatine hydrate (Eastman) are weighed on a Roller-Smith balance and added to each flask, avoiding the center cup. The water addition is made so that the final volume is 3.0 ml. In the complete system, the addition amounts to 1.5 ml. The creatine does not dissolve at this point but does dissolve when all of the reagents have been added and the flasks are in the bath at 37.5°C. The water

is followed by 0.2 ml. of 0.5 *M* KCl, 0.2 ml. of 0.1 *M* MgCl₂, 0.2 ml. of 0.25 *M* NaF, and 0.1 ml. of 0.1 *M* sodium phosphate pH 7.4, equivalent to 310 γ of inorganic P. The phosphate is added with maximum accuracy. The flasks are then placed in a pan of finely cracked ice, and the following additions are made: 0.2 ml. of 2.0×10^{-4} *M* cytochrome *c*, 0.2 ml. of 0.0133 *M* sodium adenosine triphosphate (ATP) pH 7.4, 0.2 ml. of 0.5 *M* Na succinate pH 7.4, and in the center cup, 0.1 ml. of 2 *M* NaOH plus 1 sq. cm. of folded filter paper for CO₂ absorption. One ml. of distilled water is then placed in a homogenizer tube with pestle, and the tube is placed in cracked ice. The rat is killed by decapitation and one kidney is quickly removed and placed in a beaker of cracked ice. After about 2 or 3 minutes, the chilled kidney is trimmed, the capsule removed, and the kidney weighed on the Roller-Smith balance and dropped in the homogenizer tube. The homogenization is carried out in a cold room, and ice-cold distilled water is added to give a 10 per cent homogenate, of which 0.2 ml. is added to each Warburg flask; in one flask, 2 ml. of 17.5 per cent trichloroacetic acid are added immediately, and the phosphate analysis (see below) is taken as the initial concentration of inorganic phosphate. The remaining flasks are attached to manometers, equilibrated in the 37.5° bath for 10 minutes, and oxygen uptake is measured for 10 minutes. The flasks are then removed from the bath, placed in cracked ice, and the contents precipitated with 2.0 ml. of 17.5 per cent trichloroacetic acid. The time of cooling is considered to compensate the initial time of warming, and the phosphate esterification is therefore based upon a 20 minute incubation time.

The flask contents are then transferred to 15 ml. centrifuge tubes and centrifuged in the cold room. All tubes except those for the final color development are kept in cracked ice when not in the cold centrifuge. In order to measure phosphate esterification the "true" inorganic phosphate is separated from the phosphocreatine by precipitation as follows: two ml. of the protein-free filtrate are brought to pH 8.3 (phenolphthalein) with 0.41–0.45 ml. of 2 *N* NaOH (from a paraffin-lined bottle), and 0.6 ml. of 10 per cent CaCl₂ saturated with Ca(OH)₂ are added. Each tube is centrifuged, the supernatant is poured off for the phosphocreatine analysis, and the tube is inverted, drained, blotted, and the precipitated inorganic phosphate (+ATP) is suspended in 2.0 ml. of water, dissolved by adding 0.2 ml. of 2 *N* HCl and the volume adjusted to 10 ml. with water. We determine phosphate by a micromodification of the Fiske-Subbarow method using 1 ml. of sample, 1.0 ml. of water, 0.6 ml. of 2 *N* HCl, 0.25 ml. of 2.5 per cent ammonium molybdate, and 0.15 ml. of reducer to give a final volume of 3 ml. in 13 \times 100 mm. tubes in the Cenco-Sheard spectrophotometer. The analyses are done in duplicate using 1 ml. aliquots of the calcium filtrate for the phosphocreatine determination and 1 ml. of the phosphocreatine-free solution for the inorganic phosphate determination. The results are expressed in terms of the phosphate esterified using the zero flask as the original value, which is approximately 310 γ inorganic P and about 2 γ of phosphocreatine P per flask. The former represents the inorganic phosphate added, and the latter probably represents the limiting solubility of the calcium salt precipitates, since it is not due to the tissue addition. When esterification does not take place, ATP is split, inorganic phosphate increases, and phosphocreatine formation decreases because of the diminished concentration of ATP. It is clear that the uptake reported is the net uptake remaining after compensation for the ATP breakdown. However, the presence of fluoride and

magnesium ions helps to lower the action of ATP-ase, although the action of magnesium is not simple and is also involved in the phosphorylating mechanism.

RESULTS

Components of the System. Using the method described above and the complete system acting over a period of twenty minutes with succinate as the oxidizable substrate, the original concentration of 310 γ of phosphate will be lowered to 220–260 γ of phosphate, indicating a net phosphate uptake of 50 to 90 γ (in addition to the phosphate uptake required to balance ATP breakdown). The effect of omitting certain components of the system is shown in Table I, in

TABLE I
Components of the Phosphorylation System
(Complete system and technique described in text)

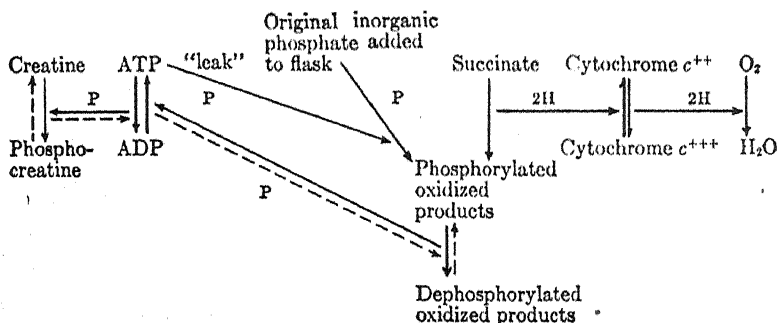
Expt. No.	Factor omitted	Micrograms P uptake per 20 minutes		Microliters Oxygen uptake per 10 minutes	
		Complete	Incomplete	Complete	Incomplete
64	Cytochrome c	86	–36*	75.5	18.0
62	Creatine	85	32	58.1	60.7
45	ATP	53	17	78.0	53.0
45a	Fluoride ions	28	–53	72.3	59.0
115	Magnesium ions	79	–96	63.7	45.6
115a	Succinate	74	–18	62.6	4.6

* A minus value indicates an increase in inorganic phosphate due to ATP breakdown outpacing ATP resynthesis.

which the “complete” systems are not all at optimum concentrations, and therefore vary slightly. Each experiment was done with a different rat. The data clearly demonstrate the necessity of adding cytochrome, creatine, ATP, fluoride, magnesium, and substrate to the reaction medium. Each of these substances was tested over a range of concentrations to select the optimum. Experiments with variation in sodium and potassium ions failed to reveal a dependence on either ion but both were retained in the final mixture. The phosphate concentration was kept low so as to increase the accuracy of the uptake measurements.

Phosphocreatine Formation. When the original reaction mixture was devised it was assumed that if creatine were present, the phosphate which was esterified during the oxidative process would be transferred

via the ATP system to creatine, forming phosphocreatine. Creatine is theoretically an ideal phosphate acceptor to use in phosphorylating systems because there is apparently no way the phosphate can be removed enzymatically except via the ATP system. These mechanisms, though quite well established in the case of muscle, have not received much attention in the case of other tissues. It was therefore desirable to determine whether phosphocreatine was formed when inorganic phosphate was esterified in a kidney homogenate in reaction mixtures such as were used to obtain the data in Table I. The data in this table show that in an experiment on the effect of creatine addition, 32 γ of phosphate were taken up in the absence of creatine and 85 γ were taken up when 30 mg. of creatine were added. In Fig. 1 are presented the data from experiments in which phosphocreatine formation was measured in addition to the determination of inorganic phosphate uptake, in reaction mixtures in which creatine was the variable. The data reveal a number of facts which lead to an understanding of the events which take place in this reaction mixture. In the first place, when no creatine was present, no phosphocreatine was formed, but some inorganic phosphate was esterified—the amount being limited by the lack of phosphate acceptors. When creatine was added, phosphocreatine was formed, and the amount of inorganic phosphate esterified was greatly increased. The dependence of the phosphorylations upon the oxidative reactions is shown by the low phosphocreatine formation and the *increase* in inorganic phosphate concentration (release from ATP) when no succinate was present. The data in Fig. 1 and Table I seem to be explainable on the basis of the following coordinated reaction mechanisms:



Further evidence that phosphocreatine was formed was obtained by observing the rate of color development in the analysis of the phosphocreatine fraction. The color increased during 20 minutes at room temperature and then remained constant. This hydrolysis rate is characteristic of phosphocreatine (20).

The data in Fig. 1 show that the optimum creatine concentration for inorganic phosphate uptake is not exactly the same as the optimum for phosphocreatine formation. A consideration of the reactions in-

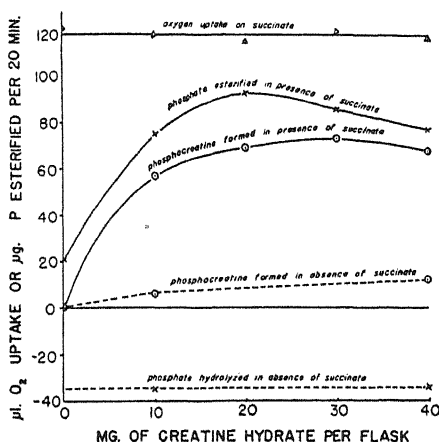


FIG. 1

The Effect of Succinate Oxidation on the Amount of Inorganic Phosphate Esterified and Phosphocreatine Formed with Varying Concentrations of Creatine in the Reaction Mixture

When no succinate was present no oxygen was taken up. Twenty milligrams of homogenized rat kidney with additions as given in the text:

involved makes it clear that for phosphate uptake the ATP:ADP ratio needs to be low, while for phosphocreatine formation it needs to be high; a perfect balance between the reactions must be attained for optimum efficiency. This balance was changed by increasing the concentration of creatine transphosphorylase through the addition of a muscle extract which had been freed from ATP-ase (Potter, unpublished), which had the effect of decreasing the difference between the phosphocreatine formation and the phosphate uptake.

The data seem to indicate that the same basic reaction mechanisms for biological energy transformations in muscle can be demonstrated in kidney homogenates. Preliminary experiments on liver, heart, skeletal muscle, and brain suggest that the basic pattern of energy transformation can be demonstrated in these tissues as well but it is already clear that each tissue differs quantitatively from the others with respect to the balance between the various enzymes. Thus liver seems to be nearly devoid of creatine transphosphorylase, while kidney has appreciable quantities of the enzyme.

Lability of the System. Having obtained phosphate esterification when precautions to avoid enzyme destruction were taken, it was possible to determine whether the precautions were necessary. The data in Table II show that the enzyme system is quite labile. In

TABLE II
Lability of the Phosphorylation System

Expt. No.	Conditions of experiment	P uptake per 20 min. micrograms	O ₂ uptake per 10 min. microliters
43	a. Complete (see text)	43	84.7
	b. Homogenate given preliminary incubation of 20 min. at 37.5°C.	-80	66.5
88	a. Complete	66	52.4
	b. Homogenate given preliminary incubation of 10 min. at 37.5°C.	-23	56.5
	c. Homogenate from kidney taken 45 min. post mortem	-36	48.0
	d. Homogenate stored 45 min. at 0°C.	49	57.8

Expt. 43, the complete reaction mixture was the same as described in the section on methods, and the results were compared with those in a similar mixture except that an aliquot of the homogenate used in the "complete" test was incubated alone for 20 minutes at 37.5°C. before being added to the other reactants. Table II shows that this treatment almost eliminated the ability of the preparation to esterify inorganic phosphate; there was no net uptake of phosphate, and enough inorganic phosphate was split from ATP to account for a loss of one high energy phosphate per mole. On the other hand, the decrease in oxidative ability was not marked. Previous experience with the succinoxidase system has shown that 20 minutes at 37.5°C. would

have little effect on succinate oxidation. In Expt. 88b it was shown that only 10 minutes at 37.5°C. sufficed to destroy the phosphorylation mechanism, while the oxidation was unimpaired. This experiment is particularly important from the technical standpoint because it indicates that the enzymes must be in a position to mobilize energy as soon as they are warmed. The usual practice of making delayed additions from a side-arm attached to the main flask is thus likely to result in a decreased enzyme activity and is not used in this work. Expt. 88 also shows that the homogenization *per se* does not destroy the enzyme systems responsible for the transformation of oxidative to phosphorylative energy; the destruction occurred in an intact kidney which was anoxic *in situ* for 45 minutes *post mortem* (88c), whereas it did not occur in a homogenate freshly prepared in the cold (88a) and did not occur to a very great extent in a homogenate kept in the cold for 45 minutes (88d). From the standpoint of shock, the experiments are interesting because they show that cellular disorganization *per se* (as illustrated by homogenization) does not destroy the enzymes; the destruction of the enzymes is due, rather, to the placing of the disorganized tissue in a situation where energy is not accessible, and where existing stores of energy are depleted. In this loss of enzyme (or vital) activity, both the elements of time and temperature (Table II) play important roles, and if energy is not available to the tissue (anoxic kidney, Expt. 88c) the destruction of the enzyme system occurs independently of cellular destruction. The phosphorylating mechanism is probably the first to be lost in the animal in shock, just as it is in a homogenate.

Time of Incubation. The earlier experiments were carried out with an incubation period of twenty minutes. Fig. 2 presents data which show clearly that during this period the phosphate uptake reaches a maximum, and that following a period in which the phosphate system is in a "steady" state in spite of a continuing oxygen uptake, the phosphate equilibrium begins to reverse its earlier trend, the phosphate is liberated into the medium. The data are from two experiments using two different animals as a source of the kidney homogenate. It can be seen that preparation A, which gave the more rapid oxygen uptake, also gave the greater amount of esterified phosphate. It is assumed that phosphate is esterified during the steady state but that breakdown and synthesis are balanced during this period. Experiments with radio-active phosphorus are needed to settle this point. All

other experiments reported in this paper were carried out with an incubation period of twenty minutes.

Substrate Experiments. The decrease in esterified phosphate which occurs during a long incubation period in a system which is oxidizing succinate suggests that perhaps the oxidation products of succinate may accumulate and act as phosphate acceptors. To test this explanation, experiments were carried out with malate and with oxalacetate

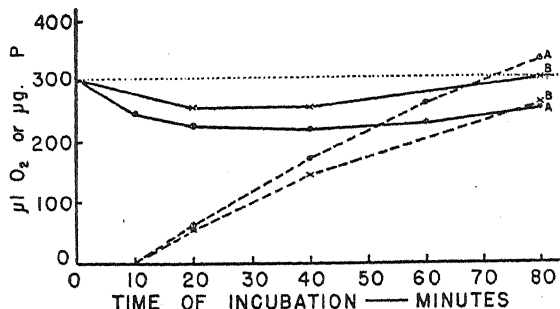


FIG. 2

Coupled Oxidation and Phosphorylation in the Succinoxidase System.
Effect of Prolonged Incubation

Experiment A represented by ○. Experiment B represented by X. Solid lines represent inorganic phosphate concentration. Dashed lines represent oxygen uptake. Experimental conditions identical but 2 different animals used.

as the oxidizable substrates, since if the proposed explanation were correct, it should be possible to demonstrate (1) that the products of succinate oxidation are not oxidized by this system as rapidly as they are formed; and (2) that when present in amounts comparable to the amounts which accumulate, inorganic phosphate is returned to the medium. It was found that both of these conditions were fulfilled. The substrate concentration used in the case of succinate had been the minimum level which gave maximum oxygen uptake and confirmed our earlier experience on this point (6). However, when malate was tested at this level, no phosphate uptake was obtained and in fact a net loss occurred. Oxalacetate gave results similar to those with malate. The effect of substrate concentration upon phosphate and oxygen uptake was then studied in the case of all three substrates over a range of concentrations from 0.00067 *M* up to 0.017 *M*, and it was found

that succinate differed in several respects from the substrates that are formed from it. The results are shown in Fig. 3, which is based upon a number of experiments. Each point represents the average of data from 2 to 6 flasks with the larger number of flasks at the critical points in the curves. The maximum oxidation rate of oxalacetate was about 27 μ l. of oxygen per 10 minutes, while malate was oxidized about half as rapidly. Since succinate is oxidized by this system at a rate of about

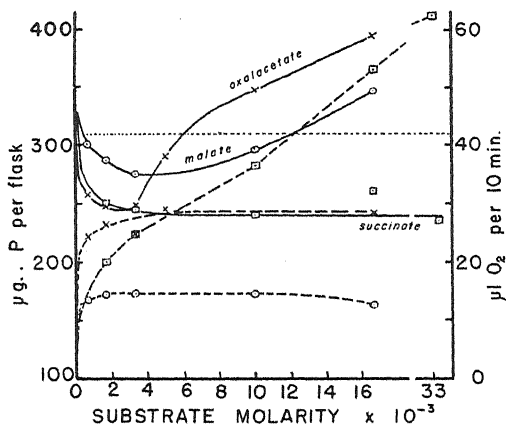


FIG. 3

Comparison of the Effect of Substrate Concentration on the Oxygen Uptake and Phosphate Esterification Using Succinate, Malate, and Oxalacetate

Solid lines represent inorganic phosphate concentration after 20 minutes. Dashed lines represent oxygen taken up in 10 minutes. Fine dotted line represents original inorganic phosphate concentration.

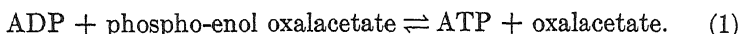
◻ = Succinate;

○ = Malate;

x = Oxalacetate

60 μ l. of oxygen per 10 minutes and since fumarase has an activity of the same order of magnitude, it is clear that malate will accumulate in this system at a rate of about 46 μ l. per 10 minutes. The effect of substrate concentration on oxidation and phosphorylation has been summarized in Table III, which is derived from the data in Fig. 3. Malate and oxalacetate give similar results while succinate differs in several respects from its products. It was thought that the decrease in net phosphate uptake over long periods of succinate oxidation might be due to phosphate acceptance by accumulated end products of succinate oxidation. Fig. 3 shows that the products could accumulate,

and it shows further that in the presence of an excess of either malate or oxalacetate, the net phosphate uptake not only decreases but that it becomes negative, that is, there is a net loss in ATP. It has been suggested by Lipmann (21) on theoretical considerations that the oxidation of malic acid results in the formation of phospho-enol oxalacetate, which could then reversibly transfer its high energy phosphate to the ATP system, according to the equation:



The fact that low concentrations of malic and oxalacetic acids result in a net phosphate uptake while high concentrations cause phosphate

TABLE III

*The Effect of Substrate Concentration on Phosphorylation and Oxidation.
An Analysis of Fig. 3*

Substrate	Effect of increasing the substrate concentration 10-fold above the range which gives optimum phosphorylation	
	Oxidation rate	Net phosphate uptake
Succinate	Increases	Remains constant*
Malate	Remains constant	Decreases
Oxalacetate	Remains constant	Decreases

* For a short period of incubation. See Fig. 2.

output, constitutes some experimental support for Lipmann's formulation, since by mass action, an excess of oxalacetate would tend to push reaction 1 to the left, removing phosphate from ATP. The phospho-enol oxalacetate, which is commonly assumed to be formed, has never been isolated, and would perhaps be analyzed as inorganic phosphate. It is also possible that enzymatic dephosphorylation of phosphoenol-oxalacetate becomes significant when the substrate concentration is raised. Fig. 3 shows that oxalacetate is much more active than malate in causing phosphate output, and malate may act *via* oxalacetate. Lardy, *et al.* (22) have observed dephosphorylation of ATP by oxalacetate in an enzyme preparation free from ATP-ase, but their enzyme was fluoride-sensitive. Succinate apparently does not accept phosphate from ATP, since an excess of succinate does not cause a loss of phosphate (Fig. 3).

It is difficult to exclude the possibility that the phosphate uptake observed when succinate is oxidized may be due to the oxidation of the malate or oxalacetate derived from succinate. Fig. 3 shows that

120 μ l. of oxygen are taken up during twenty minutes of succinate oxidation, and this corresponds to about 0.0045 *M* malate in a volume of 3.0 ml. The malate oxidizing system would thus be saturated within 5 minutes, and hence the oxalacetate system would be brought in. The phosphate uptake observed with succinate may thus be the result of the oxidation of all three substrates. It has been suggested that the oxidation of succinate to fumarate results in the esterification of one phosphate per atom of oxygen in contrast to pyruvate oxidation which was said to have a phosphate: oxygen ratio of 3 (23). The present data do not confirm or deny these ratios, but they indicate that any study which attempts to isolate succinate oxidation must take into consideration the effect of the accumulation or removal of malate and oxalacetate.

DISCUSSION

From the physiological standpoint, the studies show that the achievement of a high rate of oxygen uptake in a tissue by means of the addition of succinate is no guarantee that phosphate-bond energy is thereby provided. Tissue oxidations are of value only when they provide energy that can be used. Apparently this energy must be in the form of high-energy phosphate-bonds before it can be used for function. The succinoxidase system is very resistant to tissue damage, and it can utilize oxygen even when not accompanied by a net phosphate esterification. When the oxidation of oxalacetate is slower than the oxidation of succinate, the 4-carbon phosphate-acceptors can actually deplete the ATP reservoir and thus one can picture conditions in which the oxidation of succinate might result in a net loss to the organism. Since succinate oxidation can proceed in the absence of a net gain in phosphate-bond energy, and since the oxidation products of succinate can deplete the ATP reservoir if they are allowed to accumulate, the next logical step is the exact definition of the weak link in the chain of reactions which are secondary to succinate oxidation, that is, the oxidative removal of oxalacetate and subsequent reactions. Is the weak link in the transphosphorylation mechanism or in the oxidative decarboxylations? Since the transphosphorylation mechanism operates between the ATP system and phosphorylated intermediates in the oxidation sequence, the oxidative mechanism has to be reconstructed before the transphosphorylation mechanism can be studied. In view of the facts regarding their *in vivo* action it is not

unlikely that insulin as well as the hormones of the adrenal cortex may be directly or indirectly involved in one or the other of these enzyme mechanisms but it remains to be seen whether these hormones can be shown to act in a cell-free extract. However, the development of the techniques for the study of the coupled oxidations and phosphorylations in tissue homogenates is a necessary preliminary to any attempt to study hormone participation in these reactions. If one of these hormones could be shown to participate in these reactions *in vitro* it would perhaps be easier to define the conditions under which the factor should be used therapeutically.

SUMMARY

1. The conditions necessary for the study of oxidative phosphorylation in dilute kidney homogenates are described.

2. Succinate was found to be effective as a substrate for oxidative phosphorylation over a wide range of concentration, whereas malate and oxalacetate were effective in a very restricted concentration range (about 0.003 *M*) for phosphate uptake measurements. The data indicate that oxalacetate may accept phosphate from ATP. This observation needs to be considered in studies on oxidative phosphorylation.

3. The lability of the phosphorylating mechanism was demonstrated. It was suggested that the phosphorylating mechanism may be the first to be lost in an animal in shock as well as in a preparation of homogenized tissue. The components of the enzyme systems responsible for oxidative phosphorylation have not yet been shown to include any of the hormones whose *in vivo* action suggests that they may participate in these enzyme reactions, but the therapeutic use of these hormones should be facilitated by a knowledge of their exact locus of action. The present studies constitute a necessary preliminary to the *in vitro* study of the action of the hormones on these mechanisms.

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Growth Factor Requirements of Saccharolytic Butyl Alcohol-Acetone Bacteria

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INTRODUCTION

In a Letter to the Editors (1) we reported the growth factor requirements of three saccharolytic butyl alcohol-acetone bacteria. Further studies on these same organisms have now been completed and are considered in this paper.

The nutritive requirements of the acetone-butyl alcohol bacteria and the factors involved in the production of solvents from synthetic or semi-synthetic media have been widely investigated (2-10).

In the present work the three cultures studied have been designated B₁, B₂, and BA₁, pending their actual classification. Culture B₁ was an old stock culture, whereas cultures B₂ and BA₁ were recently isolated from soil. All three organisms attacked molasses readily but fermented corn starch slowly. B₁ and B₂ required only biotin for maximum growth and BA₁ needed both biotin and *p*-aminobenzoic acid. On the synthetic glucose-biotin medium, B₁ produced solvents in amounts which compared favorably with those from the molasses fermentation; this was considered normal. Cultures of B₂, however, gave a subnormal yield of solvents on the same medium. The BA₁ organism produced almost normal quantities of acetone and butyl alcohol from glucose when both biotin and *p*-aminobenzoic acid were present.

EXPERIMENTAL

Cultures and Inoculum. The stock cultures were maintained in sterile soil. Tubes of freshly boiled sterile potato mash were inoculated while hot with a loopful of the soil culture, allowed to stand for one minute, cooled, and incubated at 37°C. After 24 hours, a transfer was made from each tube into a medium containing 2.55% glucose and 0.45% high test cane molasses. This ratio was chosen because preliminary

* Based on data submitted in partial fulfillment of requirements for the degree of Doctor of Science in Bacteriology, University of Michigan.

experiments had shown that growth did not occur when the molasses provided less than 10% of the total sugar. The glucose-molasses culture was centrifuged after 24 hours incubation, the cells suspended in sterile distilled water, recentrifuged, and resuspended in sterile water. One-tenth ml. of this preparation was used to inoculate 10 ml. of medium in the growth requirements tests. For the fermentations, 3.0 ml. of the 24-hour glucose-molasses cultures were employed for inoculating 150-ml. portions of molasses, glucose-molasses, or synthetic medium.

Basal Medium. This was composed of 3.0% glucose, 0.3% ammonium sulfate, Speakman's salts* (1.0 ml. per 10 ml. of medium), and 0.18% calcium carbonate. Biotin as the hydrolyzed methyl ester, and *p*-aminobenzoic acid were added in quantities of 0.001 μ g. and 0.2 μ g. per ml. of medium, respectively.

Incubation and Measurement of Growth. The cultures under investigation for growth requirements were placed in a jar containing 2–3 inches of moistened oats evacuated to provide anaerobic conditions, and incubated at 37°C. The amount of sugar consumed during the period of incubation was used as an index of growth. Turbidity measurements were tried but the excess of CaCO_3 in suspension interfered with this procedure. The fermentations were carried out in 250-ml. Erlenmeyer flasks, and were freed of oxygen by bubbling nitrogen through them for 10–15 minutes. They were then sealed from the air, and allowed to incubate at 37°C.

Methods of Analysis. Sugar was determined by the Shaffer-Somogyi method (11). The iodoform reaction was used for the determination of acetone and carried out as follows: 25 ml. of 1 *N* NaOH and 25 ml. of 0.1 *N* I_2 were added to a sample containing not more than 0.35 millimoles of acetone and the temperature of the mixture was kept at 0°C. for ten minutes. The reaction was carried out in glass-stoppered bottles. The solution was acidified with an excess of 0.2 ml. of 2 *N* H_2SO_4 . The excess iodine was titrated with 0.05 *N* $\text{Na}_2\text{S}_2\text{O}_3$ using starch as an indicator. A blank on the reagents was run simultaneously. The acetone was calculated as follows:

$$\frac{(\text{Titration of blank} - \text{Titration of sample}) \times \text{Normality of } \text{Na}_2\text{S}_2\text{O}_3}{6} = \text{mM Acetone}$$

The butyl and ethyl alcohols and the volatile acids were estimated by the partition method of Osburn, Wood, and Werkman (12). The alcohol distillate was first oxidized with potassium dichromate and sulfuric acid and steam distilled.

Carbon dioxide and hydrogen, lactic acid and 2,3-butyleneglycol were determined according to the methods used by Mickelson and Werkman (13).

Cultural and Morphological Characteristics. The three organisms are obligate anaerobes and show very slight differences in morphology. They all form spores with enlargement of the cell, either terminal or subterminal. Surface colonies on glucose agar plates are circular or irregular, raised and moist.

Among the biochemical reactions studied were nitrogen fixation, hydrogen sulfide production, hydrolysis of certain proteins, nitrate reduction, and fermentation of a selected group of carbohydrates and related compounds.

* Speakman's salts: 0.25% K_2HPO_4 , 0.25% KH_2PO_4 , 0.10% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005% NaCl , 0.005% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.005% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$.

Table I gives the results of the cultural studies.

TABLE I
Biochemical Reactions of Cultures Designated as B₁, B₂, and BA₁

	Dextrose	Glycerol	Xylose	Arabinose	Rhamnose	Mannitol	Corn starch	Pectin	Casein hydrolysis	Albumin hydrolysis	Gelatin liquefaction	Nitrate reduction	Hydrogen sulfide production	Nitrogen fixation
Control	—	—	—	—	—	—	—	—	—	—	—	—	—	—
B ₁	AG	—	AG	AG	AG	AG	±	—	—	—	—	—	—	—
B ₂	AG	—	AG	AG	AG	AG	±	—	—	—	—	—	—	—
BA ₁	AG	—	AG	AG	—	—	±	—	—	—	—	—	—	—

AG — acid and gas.

± — little acid and gas.

— — no acid or gas.

Nutrient Requirements. Thiamine, riboflavin, pyridoxin, biotin, pantothenic acid, nicotinamide, inositol, and *p*-aminobenzoic acid were tested for their growth stimulating effect. A medium containing all of these growth factors in addition to glucose and salts supported growth of all three organisms. By eliminating one vitamin at a time from this medium, it was observed that only biotin was essential for strains B₁ and B₂, while BA₁ required both biotin and *p*-aminobenzoic acid. To confirm these findings the organisms were grown in combinations of the basal medium with each single vitamin. B₁ and B₂ were able to consume the sugar in the glucose-biotin mixture. BA₁ showed no growth in the medium containing just biotin or *p*-aminobenzoic acid, but utilized most of the sugar in the presence of both factors.

Table II gives the residual sugar in some of the media tested at the end of seven days. The figures are self-explanatory.

The stimulating action of biotin on the growth of these organisms was found to be inhibited or entirely suppressed by the addition of a small amount of egg white to the medium.

Fermentations. Fermentations were carried out in volumes of 150 ml. of media. The yields of solvents were determined as follows: An aliquot portion of the fermentation liquor was acidified with sulfuric acid before distillation using Congo red as indicator. This precaution was necessary because some sugar might be present. The

TABLE II

Effect of Biotin and p-Aminobenzoic Acid (= PABA) on the Growth of Cultures Designated as B₁, B₂, and BA₁

Residual sugar, in percentage, after 7 days

Culture	B.M.* only	B.M. + Biotin	B.M. + PABA	B.M. + Biotin + PABA
B ₁	2.85	0	3.13	0.2
B ₂	2.87	0	2.96	0.2
BA ₁	2.91	2.89	2.96	0.86

* Basal medium, control.

distillate which was collected in a small amount of ice-cold water, contained the volatile acids and neutral volatile products. It was subsequently neutralized with sodium hydroxide, using phenolphthalein (dry powder added) as indicator, and re-distilled. The second distillate, which contained acetone and butyl and ethyl alcohols, was collected under water in a volumetric flask which was kept in an ice bath. Aliquot portions of this neutral distillate were used for the determination of acetone and butyl and ethyl alcohols.

The residues from the two distillations were combined, sulfuric acid added to the acid end point of Congo red, and steam-distilled. The distillate contained the volatile acids, butyric and acetic. These substances were then determined quantitatively by the partition method.

When a complete analysis of the products of fermentation was desired, the technique outlined in (12) was followed. The residual carbon dioxide was first removed and weighed before the initial distillation. Lactic acid and 2,3-butyleneglycol were determined on an aliquot portion of the fermentation liquor. Lactic acid was determined on the residue of the 2,3-butyleneglycol distillation.

The results obtained when B₁, B₂, and BA₁ were cultured on cane molasses and synthetic media are shown in Table III.

TABLE III

The Yield of Solvents Produced by Strains of B₁, B₂, and BA₁, when Cultured on High-Test Molasses and Synthetic Media

Culture	Medium	Products in mM. per 100 mM. glucose fermented				
		Acetone	Butyl alcohol	Ethyl alcohol	Butyric acid	Acetic acid
B ₁	Molasses, H.T.	24.50	54.00	13.40	1.80	6.42
B ₁	Glucose-biotin	17.80	44.80	4.88	2.10	7.48
B ₂	Molasses, H.T.	18.60	58.82	11.20	7.14	7.99
B ₂	Glucose-biotin	4.47	28.77	0.00	25.41	26.46
BA ₁	Molasses, H.T.	17.55	46.90	9.90	8.19	7.56
BA ₁	Glucose-biotin-PABA	7.80	53.40	2.74	9.90	10.34

One of the strains, B₁, was cultured serially at 48-hour intervals for 150 generations on the basal medium containing glucose and biotin. Table IV gives complete analyses of fermentations of molasses and

TABLE IV

A Complete Fermentation Balance for the 78th Generation of Strain B₁ in Synthetic Medium when Cultured on High-Test Molasses and Glucose-Biotin Media

Culture	Medium	Products in mM. per 100 mM. glucose fermented									Carbon recovery	O/R Index
		CO ₂	H ₂	Acetone	Butyl alcohol	Ethyl alcohol	Acetic acid	Butyric acid	2,3-Butylene glycol	Lactic acid		
B ₁	Molasses, H.T.	193.00	95.00	19.60	56.30	6.22	5.50	4.80	7.65	—	92.0	0.96
B ₁	Glucose-biotin	204.00	138.20	19.00	45.20	10.10	23.70	6.50	6.04	1.08	94.0	0.99

glucose-biotin media by the B₁ culture. The 78th transfer in glucose-biotin medium was used as inoculum in these fermentations. A fermentation study was then made by inoculating a flask containing glucose-biotin medium with the 150th generation of culture B₁, to

TABLE V

Yield of Solvents Produced by 150th Generation of Strain B₁ in Synthetic Medium when Cultured in Glucose and Biotin

Culture	Medium	Products in mM. per 100 mM. of fermented glucose				
		Acetone	Butyl alcohol	Ethyl alcohol	Acetic acid	Butyric acid
B ₁	Glucose-biotin	1.00	21.45	17.90	32.90	47.20

observe whether or not the long period of growth on the glucose-biotin medium had altered the fermentation capacity of this strain. The data are given in Table V. The results show a subnormal yield of solvents and a correspondingly higher acidity. The amount of ethyl alcohol is also greater than usual.

DISCUSSION

Many studies have been made on the growth factor requirements of the butyl alcohol-acetone bacteria of the *Cl. acetobutylicum* type. In most instances biotin has been found essential for these organisms, but in no case has it been the only growth factor required.

The three strains under consideration in this paper are closely related as to morphological and biochemical characteristics. On the basis of their ability to ferment starch they are not identical with a strain of *Cl. acetobutylicum* in this laboratory. The three organisms are highly saccharolytic, non-proteolytic, and non-nitrogen fixing anaerobes. They all attack dextrose, arabinose, and xylose, but only two of them ferment rhamnose and mannitol. None of them can decompose pectin or glycerol.

All three organisms required biotin for optimum growth. Only one, the BA₁ strain, needed *p*-aminobenzoic acid in addition to biotin.

A comparison was made between yields of solvents in synthetic media and in a medium composed of high test cane molasses which is considered best for maximum solvent yields. Table III shows that the yield of acetone was generally lower in the synthetic medium. The difference is especially pronounced in the case of the B₂ strain which also gave a poorer yield of butyl alcohol and a higher acidity in the glucose-biotin medium. The presence of other B-vitamins in the medium had no effect on the production of solvents.

The B₁ organism was carried in the synthetic medium for as many as 150 transfers and maximum growth was maintained. However, a fermentation by the 150th generation on glucose-biotin medium gave unaccountably high acidity and less acetone and butyl alcohol. The culture was pure insofar as could be determined from subsequent platings. This finding suggests the possibility that the organism might have lost its ability to produce solvents after a rather long series of repeated transfers in synthetic medium.

SUMMARY

Three strains of saccharolytic butyl alcohol-acetone bacteria have been studied for their growth requirements and the effect of the composition of the medium on the yield of solvents. Two of these strains required only biotin for maximum growth and the third needed both biotin and *p*-aminobenzoic acid. Thiamine, riboflavin, pyridoxin, pantothenic acid, nicotinamide, and inositol were without effect.

Fermentations by these bacteria in high test cane molasses and synthetic media gave approximately the same results. Acetone production was slightly lower in the synthetic media.

One strain, B₁, was carried through 78 transfers at 48-hour intervals on the synthetic medium and still gave good solvent yields. However, after 150 transfers it no longer gave good solvent yields.

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LETTERS TO THE EDITORS

Action of HgCl_2 upon Various Activated Tyrosinase

Protyrosinase, from the diapause egg of the grasshopper, *Melanoplus differentialis*, is activated and converted into tyrosinase by a variety of reagents (1). The active enzyme, thus produced, has been shown to possess similar catalytic properties but to differ in its response to high temperatures (2). The effects of high temperatures seems related to the type of activator employed as well as to the manner in which the enzyme protein complex is broken down or transformed. HgCl_2 acts as an activator as well as an injurious agent for protyrosinase. The relative susceptibility to this compound of the tyrosinase produced by different reagents was investigated and the results compared with those obtained for high temperatures.

Methods of enzyme preparation, standardization and general manometer techniques employed were similar to those already noted (3).

The order of susceptibility to HgCl_2 by tyrosinase produced by various activators is as follows: dioctyl sodium sulfosuccinate (aerosol OT) activated enzyme > sodium dodecyl sulfate > urea > heat (70°-10'). This order of susceptibility to HgCl_2 is practically similar to that for high temperatures. One may reasonably infer that the type of activating agent apparently has some influence upon the susceptibility both to chemical and physical reagents of the active enzyme so produced.

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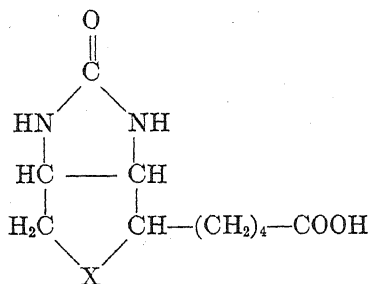
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Zoological Laboratory,
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March, 1945

J. H. BODINE

"O-Heterobiotin," a Biologically Active Oxygen Analog of Biotin

The structure of biotin, as illustrated below by a formula wherein X stands for S, suggested the preparation and biological investigation of substances in which X would be represented by other hetero-atoms, such as —O— or —NH—.



The name "*Heterobiotin*" is proposed for such biotin analogs, the *hetero-atom* in question being designated by a *prefix*.

One of the four possible racemic forms of 2'-keto-3,4-imidazolido-tetrahydrofuran-(2)-n-valeric acid, or O-heterobiotin, was prepared synthetically as the first member of this group (1). The substance crystallized from alcohol as colorless needles, melting at 205°–206°C. (cor.). Calcd. for $\text{C}_{10}\text{H}_{16}\text{O}_4\text{N}_2$: C, 52.62; H, 7.07; N, 12.27%. Found: C, 52.30; H, 6.92; N, 12.17%.

When tested as a growth factor for *Saccharomyces cerevisiae* No. 139 according to Hertz (2) and for *Lactobacillus casei* according to Shull, Hutchings, and Peterson (3), as modified by Shull and Peterson (4), the substance showed 25% of the activity of *d*-biotin for both organisms. If it is assumed that only one of the enantiomorphs composing the *dl* form is active, then its activity would be 50%. The growth-promoting activity for *Lactobacillus casei* deserves particular notice since O-heterobiotin is the only substance other than *d*-biotin reported to date which

has any significant activity for this organism. For the diaminocarboxylic acid obtained from biotin by hydrolysis, Dittmer and du Vigneaud (5) reported an activity of less than 0.01% while Stokes and Gunness (6) reported 7.2% for "this compound obtained synthetically as *dl*-diamino acid sulfate," which figure was "based on the assumption that only the *d*-form is active."

The activity of O-heterobiotin for other microorganisms and for rats, and its reaction with avidin, are being investigated.

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Research Laboratories
Hoffman-La Roche, Inc.
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April 7, 1945

R. DUSCHINSKY
L. A. DOLAN
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S. H. RUBIN.

Book Reviews

Lectures on the Inorganic Nutrition of Plants. By DENNIS R. HOAGLAND, Professor of Plant Nutrition, University of California. Waltham, Mass., The Chronica Botanica Co.; New York City, G. E. Stechert and Co. 177 pp., and 28 plates. \$4.00.

No one who is interested in plants, whether as agronomist, plant-breeder, or biochemist, can fail to appreciate the place of importance which is held by nutrition and the uptake of nutrient salts. The study of fertilizers, begun by Liebig and by Lawes and Gilbert, has developed into the studies of soil solution and soil colloids, of mineral requirements, of salt uptake and translocation, all of which have major roles in scientific agriculture. As the problems have expanded, so the methods of approach have changed greatly in the hundred years since Liebig. From the systematic but essentially simple type of field test, such as that developed at Rothamsted, to the modern laboratory with plants growing in known solutions under controlled conditions of light, temperature, humidity and atmosphere is a transition typical of all the biological sciences. To these improvements in methodology, and to the modern science of plant physiology which has resulted from them, Professor Hoagland and the group of workers at the University of California have made important contributions.

This book, based on the first series of John M. Prather lectures at Harvard University, sets out recent developments in the various fields of plant nutrition, drawing much of the material from the work of the California group. This was done, according to the author, because "the assumption was made that in lectures of the present type and objective the writer should emphasize the work with which he has had the most direct contacts. The limited scope of the lectures precluded consideration of a vast number of important contributions to the field surveyed." The result is a clear and well unified presentation, which tends, however, to avoid controversial matters which interfere with the continuity of narrative.

The California group will probably be associated most closely for the average plant physiologist, with two fields in particular. These are: the study of the microelements and the mechanism of salt absorption.

The former comprises the identification and role of those minerals required only in traces by growing plants. Although the experimental difficulties of this work are at least in inverse proportion to the quantities of minerals involved, the importance of them, as the author makes clear, is very considerable. Indeed several plant diseases of great economic influence, including Citrus mosaic, prune "die-back" and "little-leaf," have found their cures through this work. The field is an excellent example of the close interrelation between pure and applied research.

The investigations into the mechanism of salt absorption, on the other hand, have led to the emergence of a fundamental concept of "pure" plant physiology, namely the control over mineral uptake which is exerted by the processes of respiration.

This marks a transition from the physicochemical approach of de Vries and Pfeffer, based on osmotic relations, to a more truly biological viewpoint in which the different types of process in living tissue are seen to be complexly interrelated. It is probable that this part of the work of Hoagland and his collaborators will come to exert its influence not only on the applied side, *i.e.* in agriculture, but also on parallel problems in the physiology of animals.

■ The above two subjects furnish the two most interesting chapters in the book. An introduction deals with soil solutions and colloids, and three other lectures with upward movement and distribution of salts, growth of plants in artificial media, and "Some biochemical problems associated with salt absorption," respectively. The last chapter is a survey of potassium nutrition, which exemplifies the interrelationship between soil conditions, ion uptake by roots, nutritional deficiency diseases, and internal plant biochemistry.

The book is well produced, generously illustrated, and is provided with author and subject indexes. It will be indispensable to every plant scientist, and of considerable interest to a much wider circle.

KENNETH V. THIMANN, Washington, D. C.

Organic Chemistry. By LOUIS F. FIESER, Professor of Organic Chemistry in Harvard University, and MARY FIESER. D. C. Heath and Company, Boston, Massachusetts, 1944. xii + 1091 pp. Price \$8.50.

This excellent textbook by Professor and Mrs. Fieser consists of forty well-written chapters which are relatively free of typographical and factual errors.

As is stated in the preface, "the most novel feature of the book is the inclusion of a number of chapters for optional reading dealing with significant applications of organic chemistry to technology and to the biologic and medical sciences." The optional chapters include such fields as petroleum, rubber, microbiological processes, the role of carbohydrates in biological processes, the metabolism of fats, the metabolism of proteins and amino acids, quinones, polynuclear hydrocarbons, synthetic fibers, synthetic plastics and resins, steroids, accessory dietary factors, and advances in chemotherapy.

Doubtless the sections dealing with such subjects as petroleum, rubber, plastics, and vitamins will impress upon the student how deeply organic chemistry has penetrated the fabric of his daily existence and therefore make the subject more vital and interesting. One wonders, however, whether the selection and emphasis of certain other topics was not unduly influenced by the authors' own research interests.

In the chapter on accessory dietary factors, the discussion of Vitamin K requires five pages whereas the more chemically complex Vitamin B₁ is treated in only two. Three pages are devoted to the chemistry of the relatively unimportant polynuclear hydrocarbon, coronene, whereas the Skraup reaction is dismissed in two sentences in the section on dyestuffs. Conspicuously absent are chapters dealing with alkaloids and heterocyclic compounds. It may be mentioned that contrary to the authors' suggestion on page 1034, pamaquine (Plasmochin) is prepared substantially according to the patent and not by treating 6-methoxy-8-chloroquinoline with 1-diethyl-amino-4-aminopentane.

The portions of the book covering the more elementary phases of the subject are presented essentially in the conventional order with the aromatic compounds being treated in more detail than the aliphatic compounds. The authors present the material on the basis that organic chemistry is an experimental science, a practice too rarely followed by other authors. A great number of illustrative equations mention both yield and conditions and many of these examples are taken from the very recent literature. In almost every discussion consideration is given to the utility of the reaction in organic synthesis. On many occasions, the authors present in great detail the practical applications of the reactions in solving particular problems in synthesis. Many of the questions at the end of each chapter are designed to test the student's ability to solve similar problems.

The novelty of approach, the interesting manner of presenting the subject matter, the uniform excellence of the optional chapters make it a pleasure to recommend this text to all those interested in the general field of organic chemistry.

S. ARCHER, Rensselaer, N. Y.

C. M. SUTER, Rensselaer, N. Y.

A Source Book of Agricultural Chemistry. By CHARLES A. BROWNE, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture. The Chronica Botanica Co., Waltham, Mass. 1944. x + 290 pp. Price \$5.00.

This very readable book presents a detailed account of the origins and development of chemistry as applied to agriculture, beginning with the earliest recorded observations and doctrines concerning soils, plants and animals and continuing through the work of Liebig when agricultural chemistry may be said to have become a coordinated discipline. Ample space is devoted to the various authors whose contributions are discussed. Dr. Browne has adopted the excellent method of liberal quotations from these works, thus not only allowing the authors to speak for themselves but giving the reader an opportunity to know and appreciate their methods of approaching and thinking out a problem. Many of these quoted passages are models of clear, logical thinking and precise exposition. They show, among other things, that some complicated scientific thoughts can be expressed in everyday English without the assistance of "technical" terms. Another helpful feature of the book is the inclusion of numerous illustrations of the apparatus and experimental equipment used by the investigators. Many of these are not readily accessible except in the largest libraries.

But there is much better than this! The book contains Dr. Browne's critical comments none the less keen and penetrating for being phrased in the mellow kindness of his style. The criticisms of one who has long been a student of the subject and a leader among historians of science are of unusual value and will give the book a permanent place in the history of science.

The book should appeal to teachers of chemistry and agriculture, to botanists, agronomists, and soil scientists as well as to all persons interested in the development of this important field.

But two lapses have been discovered and these are of minor import: Boyle was born at Lismore Castle (p. 54), and (p. 257) "aprocrenic" acid should obviously be apocrenic.

The book is well indexed with both subject and author indices, a most useful feature in a historical work. The type and paper are good and the printing well done.

JAMES F. COUCH, Glenside, Pa.

Nutrition in Health and Disease. By LENNA F. COOPER, B.S., M.A., M.H.E., EDITH M. BARBER, B.S., M.S., and HELEN S. MITCHELL, B.A., Ph.D. Ninth Edition, Revised. J. B. Lippincott Company, Phila., 1943. xiv and 716 pp. Illustrated. Price \$3.50.

The new edition of this excellent text maintains the standards of the past. A mastery of its content will give anyone an excellent working knowledge of both the science and practice of nutrition.

The science of nutrition as it is known today has been summarized in concise form. The application of this science which falls in the domain of the dietitian, nurse, housewife, physician, dentist is presented with unusual clarity.

The seven sections of this book deal with (1) principles of nutrition, (2) diet in disease, (3) feeding of mother and child, (4) nutrition and public health, (5) food selection and cookery, (6) cooking for the sick and convalescent, (7) tabular material and special tests.

An eighth section might well have been added to discuss the importance of feeding the aged in order to help decrease the growing burden due to illness during the later years of life. Various minor additions might have been useful. The problem of adding weight to the ubiquitous descendents of Cassius might have been treated more fully. The loss of thiamin in the cooking and canning of meats as well as the loss of ascorbic acid in the preparation of salads might have been stressed.

In the section dealing with inorganic elements more attention to fluorine might have been justified. The amount of salt needed during hard work might have been stated.

The chapter dealing with gastric ulcers might have placed more stress on the avoidance of coffee with its caffeine, and cola drinks with their caffeine, sugar, phosphoric acid.

The section devoted to cookery might have given more attention to the equipment and operations in large scale feeding.

More discussion might have been given to the enrichment of flour and corn meal, the improvement of bread by the inclusion of dry skim milk, soy flour, and wheat germ. The importance of high protein intakes after serious injuries or acute febrile diseases might also have been stressed.

All of these subjects, however, represent small additions to this encyclopedic work.

CLIVE M. McCAY, Bethesda, Md.

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